THIS PAGE BLANK (USPTO)

(51) International Patent Classification 5 :		(11) International Publication Number:	**WO 94/00145**
A61K 37/02	**A1**	(43) International Publication Date:	6 January 1994 (06.01.94)

(21) International Application Number: PCT/US93/06254

(22) International Filing Date: 30 June 1993 (30.06.93)

(30) Priority data:
07/906,980   30 June 1992 (30.06.92)   US
08/026,800   4 March 1993 (04.03.93)   US

(60) Parent Application or Grant
  (63) Related by Continuation
    US   07/906,980 (CIP)
    Filed on   30 June 1992 (30.06.92)

(71) Applicants *(for all designated States except US)*: THE DOW CHEMICAL COMPANY [US/US]; 2030 Dow Center, Abbott Road, Midland, MI 48640 (US). INSTITUTE OF MOLECULAR BIOLOGY, INC. [US/US]; One Innovation Drive, Worchester, MA 01605 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : GARLICH, Joseph, R. [US/US]; 301 Southern Oaks Drive, Lake Jackson, TX 77566 (US). LYNCH, Samuel, E. [US/US]; 26 E. Street, Beverly, MA 01915 (US). PRIBISH, James, R. [US/US]; 4010 Linden Drive, Midland, MI 48640 (US).

(74) Agent: KIMBLE, Karen, L.; The Dow Chemical Company, Patent Department, P.O. Box 1967, Midland, MI 48641-1967 (US).

(81) Designated States: AU, CA, FI, HU, JP, KR, NO, NZ, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: TARGETED DELIVERY OF GROWTH FACTORS FOR BONE REGENERATION

(57) Abstract

Specific target delivery composition systems where growth factors are linked, optionally using an acid cleavable linker, to a polyaminomethylenephosphonic acid ligand and are especially suitable for site delivery to bone. When the acid cleavable linker is present, the composition is activated for the growth factors at the bone site while it remains inactive while circulating in the body. Process to make the compound and composition are also taught.

TARGETED DELIVERY OF GROWTH FACTORS FOR BONE REGENERATION

This invention relates to specific target delivery systems; namely, delivery of growth promoting factors to bone for its regeneration by a chelant composition system. Compositions containing these systems and a process for making them are also part of this
5   invention.

Historically, many physical conditions and diseases exist which cause bone loss in mammals, e.g., intentional and accidental traumatic injuries, osteoporosis and periodontal diseases. Thus it is often desired in the medical and dental fields to provide a composition which will stimulate and enhance bone regeneration in a mammal, e.g. a human patient.

10   One class of proteins which may be useful for regeneration of bone is polypeptide growth factors (GF), which are also described as tissue growth promoting factors. Growth factors are polypeptides which stimulate a defined population of target cells. As multifunctional molecules, they may stimulate or inhibit cell proliferation as well as affect cell function, depending on the type of the target cells and the presence of other signal peptides.

15   Examples of growth factors are platelet-derived growth factors (PDGF's), insulin-like growth factors (IGF's), transforming growth factors (TGF) such as beta's (TGF-ß's) and alpha (TGF-α), epidermal growth factor (EGF), fibroblast growth factor (FGF's) including acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF), nerve growth factor (NGF), and bone morphogenetic proteins (BMP's), including osteogenic and osteoinductive factors.

20   Combinations of tissue growth factors may be beneficial for promoting bone regeneration. For example the combinations of PDGF and IGF-I or PDGF and IGF-II promote bone regeneration and wound healing [See, for example, Lynch et al., *Proc. Nat'l. Acad. Sci.* (USA) 84, 7696-7700 (1987); Lynch et al., *J. Clin. Invest.* 84, 640-646 (1989); Lynch et al., *J. Clin. Periodontol.* 16, 545-588 (1989); Lynch et al., *J. Periodontol.* 62, 458-467 (1991); and US Patents
25   4,861,757 and 5,019,559].

PDGF's are polypeptides of about 28-35 kilodaltons (kD). They are found in numerous cell types in the body. PDGF derived from human platelets contains two polypeptide sequences, PDGF-A and PDGF-B polypeptides [See H.N. Antoniades and M. Hunkapiller *Science* 220, 963-965 (1983)]. PDGF-A is encoded by a gene localized in chromosome 7 [C. Betsholtz et
30   al., *Nature* 320, 695-699 (1986)], and PDGF-B is encoded by the *sis* oncogene [R. Doolittle et al., *Science* 221, 275- 277 (1983); Waterfield et al., *Nature* 304, 35-39 (1983)] localized in chromosome 22 [R. Dalla-Favera, *Science* 218, 686-688 (1982)].

Because the two polypeptide chains of PDGF are encoded by two different genes localized in separate chromosomes, human PDGF occurs in three forms, a disulfide-linked
35   heterodimer of PDGF-A and PDGF-B, or two different homodimers (homodimer of PDGF-A and homodimer of PDGF-B). The role of PDGF in bone formation is not clear. Some studies have indicated that it promotes bone resorption [Tashjian et al., *Endocrinology* 111, 118-124 (1982); Canalis et al., *J. Cell Physiol.* 140, 530-537 (1989)]. Other studies have shown that PDGF

-1-

stimulates the proliferation of osteoblasts *in vitro* and, when given via repeated subperiosteal injections in newborn rats, new bone formation *in vivo* [Piche and Graves, *Bone* 10, 131-138 (1989); Joyce et al., in <u>Clinical and Experimental Approaches to Dermal and Epidermal Repair: Normal and Chronic Wounds</u>, pp. 391-416 (1991).

5   IGF's, or somatomedins, are polypeptides of about 7.5 kD that have a strong homology to human proinsulin [Humbel, *Hormonal Proteins and Peptides* 12, 57-79 (1984)]. IGF-I and IGF-II share a 62% sequence homology. Their actions are mediated through two distinct receptors. The IGF-I receptor is named type-I receptor (IGF-IR), and the IGF-II receptor is named type-II receptor (IGF-IIR).

10   IGF-I by itself has also been extensively studied for its effects on bone growth. *In vivo*, the continuous local application of IGF-I inside a titanium chamber implanted into the adult rabbit tibia did <u>not</u> significantly alter bone formation [Aspenberg et al., *Acta Orthop. Scand.*, 60, 607-10 (1989)]. Continuous systemic administration of somatomedic-C (IGF-I) also failed to promote the repair of bone wounds resulting from a femoral osteotomy in rats

15   [Kirkeby and Ekeland, *Acta Orthop. Scand.*; 61, 335-38 (1990)]. A preliminary study in a small number of animals suggested that continuous infusion of IGF-I into the arterial supply of one hind limb for 14 days resulted in increased cortical bone formation in that limb in older, but not young, rats. The action appeared to be the result of an increased number of osteoblasts and decreased number of osteoclasts [Spencer et al., *Bone* 12, 21-26 (1991)]. The local application

20   of IGF-I to the growth plate of young hypophysectomized rats resulted in a small but significant effect on unilateral longitudinal bone growth [A. M. Isgaard et al., *J. Physiol.* 250, E367-372 (1986)]. IGF-I and PDGF have also been isolated from bone matrix [Hauschka et al., *J. Biol. Chem.* 261, 665-74 (1986); and Canalis et al., *Cal. Tiss. Internatl.* 43, 346-51 (1988)].

In vitro, there are apparently conflicting data on the effects of IGF-I on bone cells.

25   Pfeilschifter, et al., [*Endocrinology* 127, 69-75 (1990)] reported only a modest effect of IGF-I alone on bone matrix apposition in cultured fetal rat calvarial. Significant effects on bone matrix formation were seen when IGF-I was combined with PDGF-BB, TGF-β, or both PDGF-BB and TGF-β. In contrast, McCarthy et al., [*Endocrinology* 124, 301-7 (1989)] reported that IGF-I and IGF-II stimulate significant DNA and collagen synthesis in bone cultures. Hock et al.,

30   [*Endocrinology* 122, 254-60 (1988)] found that IGF-I stimulates primarily pre-osteoblast replication *in vitro* and that collagen and bone matrix synthesis is stimulated independently of cell replication. Canalis et al., [*J. Cell. Physiol.* 140, 530-537 (1989)] reported that PDGF-BB opposed the stimulatory effect of IGF-I on collagen synthesis, IGF-I prevented the PDGF effect on collagen degradation and that PDGF-BB and IGF-I had additive effects on calvarial DNA

35   synthesis. Piche and Graves [*Bone* 10, 131-8 (1989)] also reported that *in vitro* IGF-I did not stimulate significant 3H-thymidine incorporation into bone derived cells nor did it enhance the activity of PDGF in this regard. IGF-I in combination with PDGF, EGF and TGF-B resulted in uptake by the bone cells nearly equal to that achieved by 10% fetal bovine serum. Receptors

for IGF-I and II have been demonstrated in osteoblast-enriched cultures from fetal rat bone [Centrella et al., *J. Cell Biol.* (abstract) 107, 62a (1988)]. The role of IGF-I in bone metabolism has been reviewed recently by Canalis et al., [*J. Endocrinol. Invest.* 12, 577-84 (1989)].

5   Another class of growth factors which have been studied for their effect on bone growth is FGFs. *In vivo*, both aFGF and bFGF and their respective mRNA's have been detected at the site of bone fractures [Joyce et al., (1991) *ibid*]. Both aFGF and bFGF have been isolated from bone matrix [Hauschka et al., (1986) *ibid*]. bFGF and IGF-I have been used in combination to promote the healing of skin wounds [Lynch et al., *J. Clin. Invest.* (1989) *ibid*].

       In vitro, bFGF did not significantly alter ^{3}H-thymidine incorporation in bone
10   fracture calluses [Joyce et al., (1991) *ibid*]. bFGF has been reported to enhance mitogenesis in fetal calvarial bone cultures but did not simulate differentiated function of osteoblasts directly [Canalis et al., *J. Clin. Invest.* 81, 1572 (1988)]. aFGF has the same reported biological effects on bone as bFGF but generally requires higher concentrations [Canalis, *J. Clin. Invest.* 79, 52-58 (1987)]. Both aFGF and bFGF tend to decrease matrix synthesis in the fetal rat calvarial model
15   [Canalis et al., (1989) *ibid*]. Cultured bovine bone cells synthesize both bFGF and aFGF and store them in their extracellular matrix [Globus et al., *Endocrinology* 124, 1539 (1989)]. bFGF has been reported to enhance the capacity of bone marrow cells to form bone-like nodules *in vitro* [Noff et al., *F.E.B.S. Letters* 250, 619-21 (1989)]. Both aFGF and bFGF increased DNA synthesis in cells cultured from parietal bones while bFGF was a more potent stimulator of alpha 1 Type 1
20   procollagen mRNA [McCarthy et al., *Endocrinology* 125, 2118-26 (1989)]. They are both mitogenic and chemotactic for cells derived from the periodontal ligament and bind to pretreated dentin slabs [Terranova et al., *J. Periodontol.* 60, 293-301 (1989); Terranova et al., *J. Periodontol.* 58, 247-257 (1987); Terranova, In The Biological Mechanisms of Tooth Extraction and Root Resorption, Davidovitch Z. ed.; pp. 23-34 (1989)].

25   The TGF-β family of proteins also appear to have potential as modulators of bone growth. *In vitro* TGF-β is produced by osteoblasts and stimulates proliferation and collagen synthesis by these cells [Robey et al., *J. Cell Biol.* 105, 457-463 (1987); Rosen et al., *Exper. Cell Res.* 165, 127-138 (1986); Hock et al., *Cal. Tissue Int.* 32, 385 (abstract) (1988)]. *In vivo* injections of TGF-β stimulate chondrogenesis and osteogenesis [Joyce et al., *J. Cell Biol.* 110, 2195-2207
30   (1990); Noda et al., *Endocrinology* 124, 2991-2996 (1989)].

       Bone inductive factors, such as bone morphogenetic proteins, osteogenin and osteoinductive protein 1, can also stimulate bone formation. They are often strucurally similiar to TGF-β and are characterized by their ability to induce ectopic cartilage and bone formation when implanted subcutaneously or intramuscularly in mammals (US Patents 4,877,864;
35   4,619,989; 4,455,256; 4,596,574; and 4,563,489; Wozney et al., *Science* 242, 1528-1534 (1988)].

Targeted Delivery

       While growth factors appear to have the ability to modulate bone growth, their efficacy as therapeutic agents is limited by the ability to deliver them to the site of bone deficit.

In diseases such as osteoporosis this deficit occurs at various times throughout the entire skeletal system. Thus it would be ideal to target the delivery of the growth factors to skeletal tissue with preference given to the site of the bone deficit. Current methods for delivery of proteins, such as, polymers, bone grafts and liposomes are unsatisfactory because they allow

5 only localized delivery or systemic distribution without targeting.

Various aminophosphonic and aminocarboxylic acid complexes, having a metal complexed to an aminophosphonic or aminocarboxylic acid ligand, are known to deliver agents to bone [US Patents 4,508,704, 4,515,767, 4,560,548, 4,606,907, 4,897,254, 4,898,724, 5,059,412, 5,064,633, and 5,066,478].

10 Clearly, it would be desirable to deliver preferentially to the bone site the desired growth factor(s) and retain its activity for the intended use. More preferred would be the ability to deliver the desired growth factor(s) preferentially to the site of injured or depleted bone and still retain its intended activity. Most preferred would be the ability to deliver the desired growth factor(s) preferentially to the site of injured or depleted bone and allow for the

15 activation of the bone growth factor(s) on an "as needed" basis as determined by the natural bone homeostasis mechanisms in the body. The present invention has the ability to met the above objectives.

Present Invention

The present invention provides compounds for stimulating and enhancing bone

20 growth by administering bone growth promoting factors which have been modified by being associated with a polyaminomethylenephosphonic acid ligand in a way that allows their preferential localization to skeletal tissue. The compounds for this delivery system are represented by the formula

25 $$GF\text{-}[(CL)_z\text{-}L\text{-}AP]_q \quad (I)$$

wherein: GF is a growth promoting factor or combinations thereof;

CL is an acid cleavable linker which is covalently bonded to GF;

z is 0, 1 or 2;

30 q is from 1 to the sum of the amino groups present on the native GF;

L is a linking moiety; and

AP is a polyaminomethylenephosphonic acid ligand.

Formulations for administering the compounds of Formula I to mammals, and methods for the use of the compounds of Formula I for targeted delivery to bone, and

35 processes for preparing the compounds of Formula I are also contemplated by this invention.

When the compositions or formulations containing compounds of Formula I are used, sites of injured or depleted bone are treated, and bone regenerated. Natural and recombinant tissue growth factors are commercially available from R&D Systems (Minneapolis,

-4-

MN), Collaborative Research, Inc. (Bedford, MA), Genzyme, Inc (Cambridge, MA), ICN
Biomedicals, INC., (Cleveland, OH), Peprotech, Inc. (Rocky Hill, NJ) and UBI (Lake Placid, NY).
Bone inductive proteins can be purified from bone [Celeste et al., *Proc. Natl. Acad. Sci.* (USA) 87,
9843-9847 (1990); Wang et al., *Proc. Natl. Acad. Sci.* (USA) 85, 9484-9488 (1988); US Patents
5 4,455,256 and 4,619,989].

In the compounds of Formula I, although any of the growth promoting factors
(GF) mentioned before may be used, preferably GF is chosen from PDGF's, IGF's, FGF's, TGF's or
cartilage/bone inductive factors (BMP's).

PDGF, preferably in combination with IGF-I, has been shown to increase new bone
10 formation when applied either alone or directly to diseased bone (US Patents 4,861,757 and
5,019,559 and copending US application Serial No. 582,332, filed September 13, 1990, H.
Antoniades and S. Lynch). PDGF contains 30 free amino groups per molecule which can
potentially be modified to increase PDGF's affinity for bone. PDGF is available from R&D
Systems and Genzyme, Inc. PDGF has been described in US Patents 4,861,757 and 5,019,559.

15 IGF-I has been shown to increase new bone formation when applied, either alone
or preferably in combination with PDGF, directly to diseased bone. IGF-I contains 4 free amino
groups per molecule which can potentially be modified to increase PDGF's affinity for bone.
IGF-I is available from R&D Systems and Genzyme, Inc. IGF-I is described by R. E. Humbel, *Eur. J.
Biochem.* 190, 445-462 (1990).

20 IGF-II has been shown to increase new bone formation when applied directly to
diseased bone. IGF-II contains 2 free amino groups per molecule which can potentially be
modified to increase PDGF's affinity for bone. IGF-II is available from R&D Systems and
Genzyme, Inc. IGF-II is described by R. E. Humbel, *Eur. J. Biochem.* 190, 445-462 (1990).

bFGF has been shown to increase new bone formation when applied directly to
25 diseased bone. bFGF contains 15 free amino groups per molecule which can potentially be
modified to increase PDGF's affinity for bone. bFGF is available from R&D Systems and
Genzyme, Inc. bFGF is described by Gospodarowicz et al., *Endocrinol. Rev.* 8, 95-114 (1987).

aFGF is a low molecular weight homodimer polypeptide which has been shown to
increase new bone formation when applied directly to diseased bone. aFGF contains 14 free
30 amino groups per molecule which can potentially be modified to increase PDGF's affinity for
bone. aFGF is available from R&D Systems and Genzyme, Inc. aFGF is described by
Gospodarowicz et al., *Endocrinol. Rev.* 8, 95-114 (1987).

TGF-β_1 is a low molecular weight (about 25 kDa, amino acids) homodimer
polypeptide which has been shown to increase new bone formation when applied directly to
35 diseased bone. TGF-β_1 contains 18 free amino groups per molecule which can potentially be
modified to increase PDGF's affinity for bone. TGF-β_1 is available from R&D Systems and
Genzyme, Inc. TGF-β_1 is described by Sporn et al., *J. Cell Biol.* 105, 1039-1045 (1987).

-5-

BMP's have been shown to increase new bone formation when applied within mesenchymal tissues. The BMP's have been reviewed by Celeste et al. [*Proc. Natl. Acad. Sci.* (USA) <u>87</u>, 9843-9847 (1990)] and found to be as follows:

BMP

BMP	No. of Free Amino Groups	kDa approximate
2	10	12.9
3	11	14.5
4	8	13.1
5	11	15.6
6	8	15.7
7	9	15.7

The polyaminomethylenephosphonic acid ligands (AP of Formula I) are either covalently bonded to the GF of Formula I ($z = 0$), or have a cleavable linker (L) present ($z = 1$). The AP ligands may be straight or branched-chain moieties, cyclic moieties, polymers (including dense star polymers, their dendrimers and dendrons), or aryl moieties, which ligands contain at least two, preferably three or more, nitrogen atoms. Preferably, the ligands are polyamino-methylenephosphonic acid ligands of one of the formula
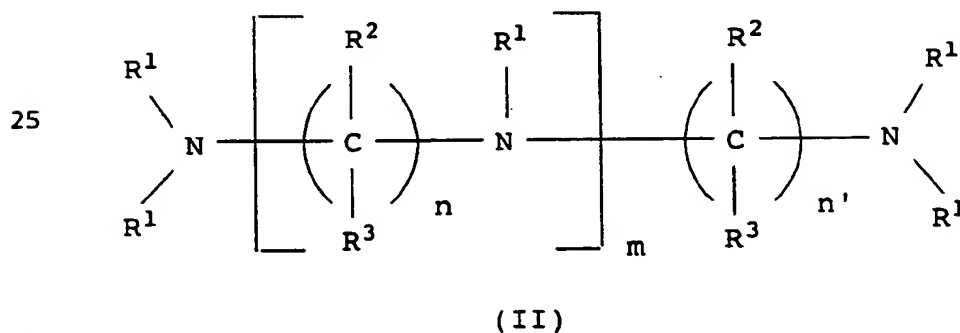
(II)

wherein:

each R^1 independently is hydrogen, C_1-C_4 alkyl, phenyl, hydroxy C_1-C_4 alkyl, -CH_2COOH, -$CH_2PO_3H_2$ or an L moiety;

with the proviso that only one of R^1 may be an L moiety and one L moiety must be present and with the proviso that at least one-half of the total R^1's are -$CH_2PO_3H_2$;

each R^2 and R^3 independently is hydrogen, C_1-C_2 alkyl or L moiety;

with the proviso that only one L moiety is present in Formula II;

-6-

n is independently 2, 3 or 4;

n' is independently 2, 3 or 4; and

m is 0 to 10; or

5

10

15

$$\left[\begin{array}{c} R^2 \\ | \\ \left(\, C \,\right)_n \\ | \\ R^3 \end{array} \begin{array}{c} R^1 \\ | \\ N \end{array}\right]_m$$

(III)
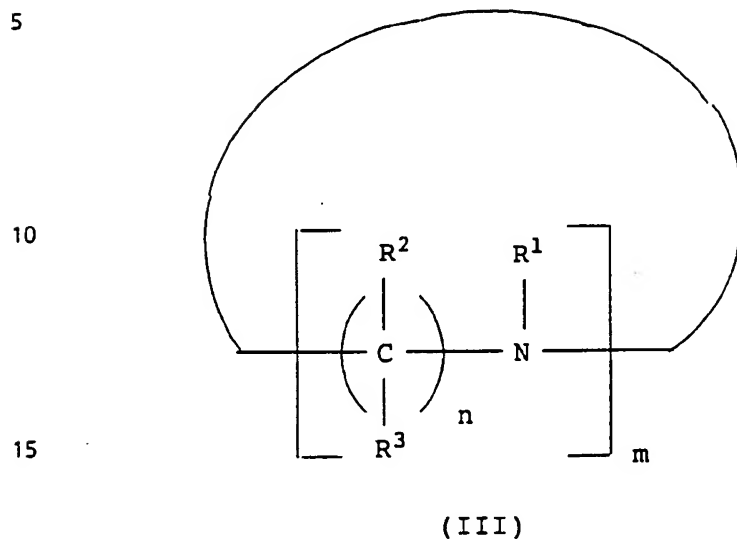
20   wherein: R^1, R^2, R^3, n and m are defined as before; or

25

30

$$R^1 — N \qquad N — R^1$$
$$N$$
$$|$$
$$R^1$$

(IV)
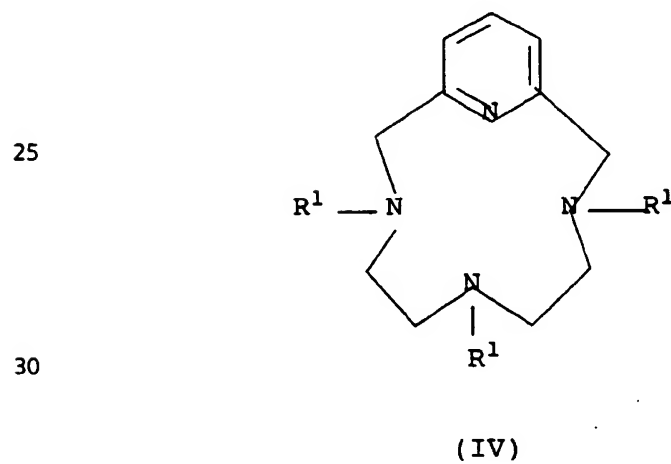
35   wherein: R^1 is defined as before.

The linking moiety (L in Formula I) is represented by the formula
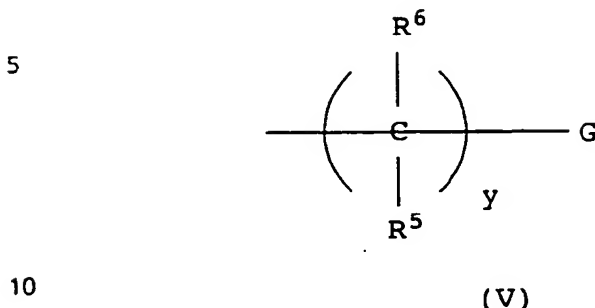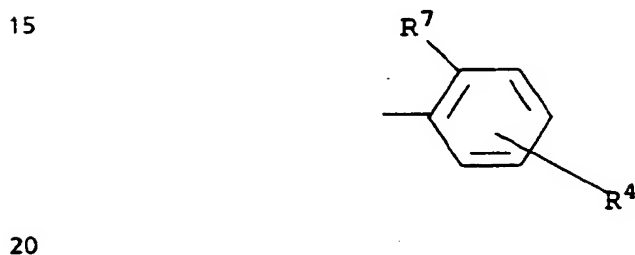
5

$$\left(\underset{\underset{R^5}{|}}{\overset{\overset{R^6}{|}}{C}}\right)_y - G$$

10   (V)

wherein: G is hydrogen, NH_2 or

15

20

R⁴ is an electrophilic group capable of being attached to protein;

R⁵ and R⁶ are independently hydrogen or -COOH;

with the proviso that when G is hydrogen, then one of R⁵ or R⁶ is COOH;

25   R⁷ is hydrogen, hydroxy or C_1-C_4 alkoxy; and

y is 0, 1, 2, 3 or 4;

with the proviso that when y is 1, 2, 3 or 4, then only one of R⁵ or R⁶ may be COOH.

In the present invention the following terms are defined as follows. The term "straight or branched-chain moieties" refers to an alkyl group having from 1 to about 100

30   carbon atoms which may be either a straight-chain moiety such as, for example, ethyl, propyl, n-butyl, n-dodecane and the like, or a branched-chain moiety such as, for example, isopropyl, tert-butyl, 2,5,7-trimethyldodecyl and the like. Both the straight and branched-chain moieties must contain at least 2 nitrogen atoms, preferably from 3 to 50 nitrogen atoms, and more preferably from 3 to 25. Some examples of these moieties include

35

-8-

PDTMP

$$HOOC - (CH_2)_3 - N \begin{cases} PO_3H_2 \\ PO_3H_2 \end{cases}$$

$$H_2O_3P$$

APEDTMP

$$H_2N - \bigcirc - CH_2CH_2 - N - CH_2CH_2 - N \begin{cases} PO_3H_2 \\ PO_3H_2 \end{cases}$$

$$H_2O_3P$$

CEDTMP

$$HOOC$$

$$H_2O_3P - N - CH - CH_2 - N \begin{cases} PO_3H_2 \\ PO_3H_2 \end{cases}$$

$$H_2O_3P$$

ABEDTMP

$$H_2N - \bigcirc$$

$$H_2O_3P - N - CH - CH_2 - N \begin{cases} PO_3H_2 \\ PO_3H_2 \end{cases}$$

$$H_2O_3P$$

35

APIPTMP

$$H_2N-\bigcirc-N\left[N\begin{array}{l}-PO_3H_2\\[1em]-PO_3H_2\end{array}\right]_2$$

APDTMP

$$H_2N-\bigcirc-CH_2CH_2-N\left[N\begin{array}{l}-PO_3H_2\\[1em]-PO_3H_2\end{array}\right]_2$$

ABDTMP

$$H_2N-\bigcirc-CH_2-\begin{array}{c}N\begin{array}{l}-PO_3H_2\\-PO_3H_2\end{array}\\N\end{array}$$

$$H_2O_3P-NN\begin{array}{l}-PO_3H_2\\-PO_3H_2\end{array}$$
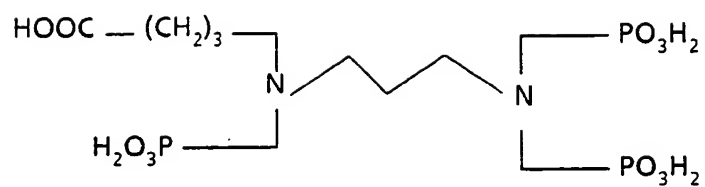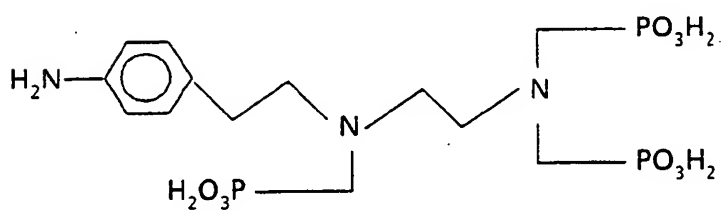
25   wherein:

PDTMP = (N-propylcarboxyl)ethylenediamine-N,N',N'-trimethylenephosphonic acid;

APEDTMP = [N-(4-aminophenyl)ethyl]ethylenediamine-N,N',N'-trimethylene-phosphonic acid;

CEDTMP = 1-(carboxyl)ethylenediamine-N,N,N',N'-tetramethylenephosphonic
30   acid;

ABEDTMP = [1-(4-aminobenzyl)]ethylenediamine-N,N,N',N'-tetramethylene-phosphonic acid;

APIPTMP = N-(4-aminophenyl)-N,N-bis-[propyl(iminodimethylenephosphonic
35   acid)];

APDTMP = N-[(4-aminophenyl)ethyl]-N,N-bis-[ethyl(iminodimethylene-phosphonic acid)]; and

-10-

ABDTMP = N-[1-(4-aminobenzyl)-N,N'-ethylenediamine-N',N''-ethylenediamine-N,N,N',N''-pentamethylenephosphonic acid;

with ABEDTMP and ABDTMP being preferred. The compounds are shown in the Examples.

The term "cyclic moieties" refers to aliphatic, saturated ring systems having at

5　least 2 nitrogen atoms, preferably from 3 to 10, and more preferrably from 3 to 8; and about twice as many carbon atoms present as nitrogen atoms. Some examples of these moieties include 1,4,7,10-tetraazacyclododecane, 1,5,8,12-tetraazacyclotetradecane, 2-[(4-amino-benzyl)-1,4,7,10-tetraazacyclododecane]-1,4,7,10-tetramethylenephosphonic acid, 1-[(α-carboxyl)-4-amino-2-methoxybenzyl]-1,4,7,10-tetraazacyclododecane-4,7,10-tri-

10　methylenephosphonic acid, and 1-[(α-phosphonyl)(4-aminophenyl)ethyl]-1,4,7,10-tetra-azacyclododecane-4,7,10-trimethylenephosphonic acid.

The term "aryl moieties" refers to an aromatic ring system which may have one or more additional cyclic or aromatic rings or substitution by straight or branched-chain moieties. The total number of atoms in the backbone of the aryl ring is from 3 to 30, preferably from 6 to

15　16, and more preferably from 8 to 16. The aryl moiety contains at least 2 nitrogen atoms, preferably from 3 to 10, and more preferrably from 3 to 8. Some examples of these moieties include pyrazolyl, 3-met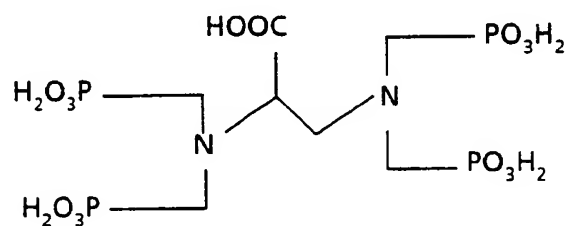hylpyra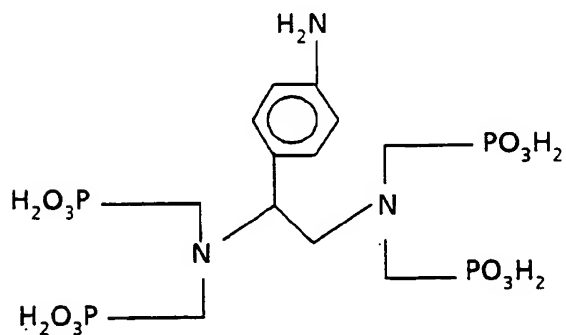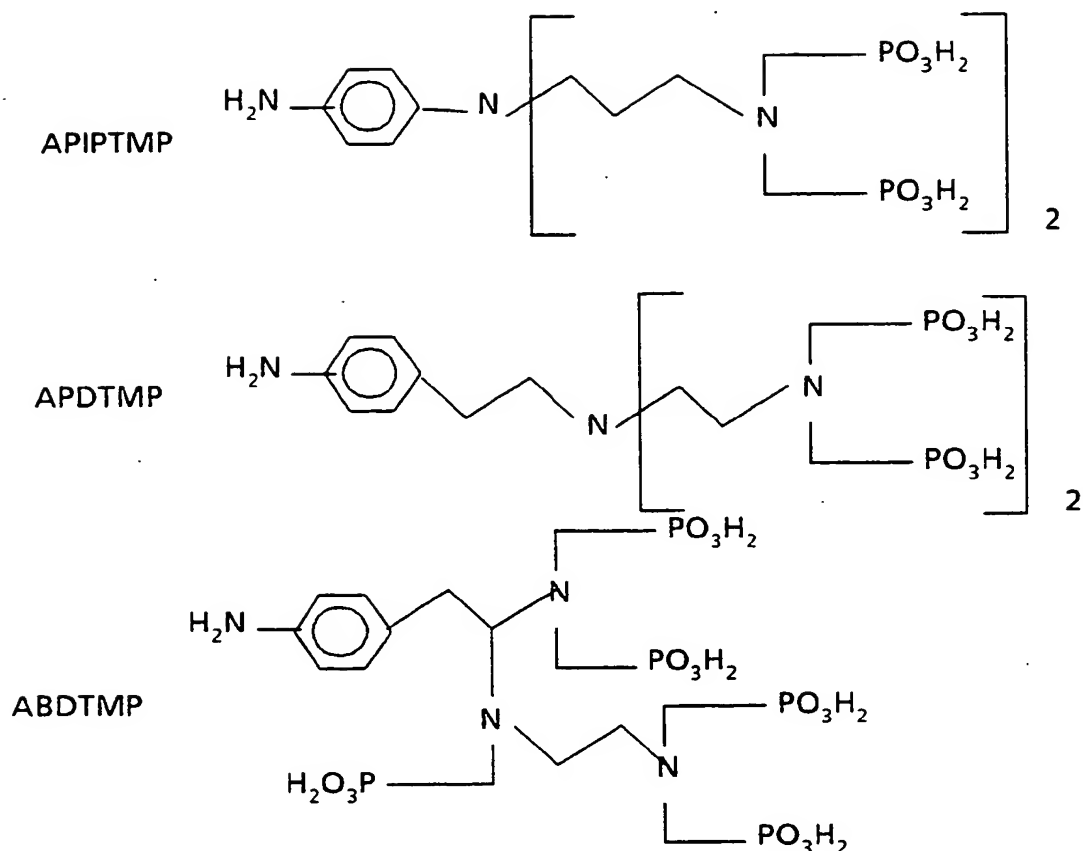zolyl, 5-methylpyrazolyl, imidazolyl, 4-methylimidazolyl, 5-methylimidazolyl, 1,4-dimethylimidazolyl, 1,5-dimethylimidazolyl, pyridazinyl, pyrimidinyl, 2,4,6-trimethylpyrimidinyl, pyrazinyl, purinyl, pterdinyl, 3,6,9,15-tetraazabicyclo[9.3.1]penta-

20　deca-1(15),11,13-triene-3,6,9-trimethylenephosphonic acid (PCTMP), 6-(α-carboxyl-4-amino-benzyl)-3,6,9,15-tetraazabicyclo[9.3.1]pentadeca-1(15),11,13-triene-3,9-dimethylenephos-phonic acid (PCAPCDMP), 13-(4-aminobenzyl)-3,6,9,15-tetraazabicyclo[9.3.1]pentadeca-1(15),11,13-triene-3,6,9-trimethylenephosphonic acid (PCABTMP), and 6-[(α-phosphonyl-4-aminophenyl)ethyl]-3,6,9,15-tetraazabicyclo[9.3.1]pentadeca-1(15),11,13-triene-3,9-

25　dimethylenephosphonic acid (PCAPCTMP). PCTMP, PCAPCDMP and PCAPCTMP, with PCTMP and PCAPCTMP preferred, and are taught in Kiefer et al.'s copending US Patent Application Serial No. 805,551, filed December 10, 1991 (assigned by unrecorded assignment to The Dow Chemical Company), the disclosure of which is hereby incorporated by reference. Other hetro atoms such as oxygen may be present.

30　The term "polymers, including dense star polymers" are defined as described in European Appln. 0 271 180, published June 15, 1988, the disclosure of which is hereby incorporated by reference. These "dense star polymers" are also referred to as STARBURST™ polymers (a Trademark of Michigan Molecular Institute, Midland, MI) or STARBURST™ dendrimers and described in the same European publication. Preferred STARBURST™

35　dendrimers have polyaminoimine groups where the surface has the amino groups converted to aminomethylenephosphonic acid groups and with at least one 4-aminophenyl moiety on the surface. These STARBURST™ dendrimers are prepared as described in European Appln. 0 271 180, published June 15, 1988, the disclosure of which is hereby incorporated by reference.

-11-

The "polymers" also include arborols (G. R. Newkome, *J. Org. Chem.* 50, 2004-2006 (1985), and either linear or branched polymer containing amines (V. P. Torchilin et al., *Byull. Eksp. Biol. Med.* 102, 63-65 (1986).

Also included within the definition of the AP term of Formula I is

5    $H_2N-(CH_2)_n-C(PO_3H)_2OH$

where n is from 1-3.

Such compounds are described in US Patents 5,039,819, 5,019,651, 4,922,007, 4,621,077, 4,134,969, 4,117,086, 4,108,962, and 3,962,432.

The term "cleavable linker" (CL of Formula I) means that the linkage between the
10   polyaminomethylenephosphonic acid (AP of Formula I) [having a bifunctional chelanting agent (BFCA) on the linking moiety (L of Formula I)] and the protein of the GF is reversible or cleavable under certain physiological conditions. Such linkages are taught in the art and include linkages containg thiourea, thioether, peptide, ester, and difulfide groups [C. F. Meares et al., *Int'l. J. Cancer,* Supp. 2, 99-102 (1988) and references contained therein, US Patent
15   5,045,312]. Also taught in the art are linkages between molecules and proteins which contain an amidine linkage. These linkages are prepared by reacting a molecule containing an imidoester with the amine groups of the protein [O. R. Zaborsky, Immobilized Enzymes in Food and Microbial Processes, p187-202, A. C. Olson and C. C. Cooney, eds., Plenum, New York (1974)]. Similarly taught in the art are amide, diester, thioether, hydrocarbon, and disulfide
20   linkages [C. H. Paik et al., *J. Nucl. Med.* 30, 1693-1701 (1989) and M. K. Haseman, *Eur. J. Nucl. Med.* 12, 455-460 (1986)]. Cleavable diphosphonate and amidated diphosphonate linkers are taught in US Patent 5,094,848. These linkers are cleaved *in situ* by enzymes such as phosphodiesterase, 5'-nucleotidase, and acid phosphatase. Cleavable linkages are also taught in US Patent 5,094,849 in the form of an alkylidene hydrizide bond. These linkages are formed
25   by reacting a carbonyl containing molecule with a molecule containing a hydrazide moiety. Additionally, acetal glycosides have been proposed as selectively cleavable linkages [L. F. Tietze, *Nachr. Chem.*, Tech. Lab. 36, 728-737 (1988)]. Linkers that are acid cleavable may be particularly advantageous. Especially preferred are acid cleavable linkers wherein their rate of cleavage is at least ten-fold greater at pH 5 compared to their rate at pH 7; both rates are determined at
30   37°C. Some examples of acid cleavable linkers are disclosed in US Patents 4,542,225 and 4,618,492 and the references mentioned therein and our copending application US Serial No. 026,800, filed March 4, 1993, which is hereby incorporated by reference. In this latter paending application, the preferred acid cleavable linker is 4-isothiocyanatophthalic anhydride.

The term "group present to permit coupling to a protein" or "electrophilic
35   group(s) capable of being attach d to protein(s)" refers to an electrophilic group (R^4 of the L term of Formula I) that can bind to an amino acid of a protein, e.g. the GF. Some examples known to those skilled in the art of suitable groups include, but are not limited to, amino, maleimido, diazo, isothiocyanato, vinylpyrido, bromoacetamido, carboxyl, and N-hydroxy-

succinimido active ester. When these electrophilic groups are present, the ligand (L-AP of Formula I, prior to attachment) is a BFCA.

The "polyaminomethylenephosphonic acid moieties" (AP of Formula I) are represented by a wide variety of possible groups, such as the cyclic moieties, straight or

5 branched-chain moieties, aryl moieties, polymers including dense star polymers, as defined above, which have at least one portion of the moiety containing between two nitrogen atoms a methylene ($-CH_2-)_n$ group where n is 2, 3 or 4 (polyaminomethylene group). More than one such polyaminomethylene group may exist in the moiety. The moiety also contains at least two methylenephosphonic acid group ($-CH_2-PO_3H_2$) covalently attached to the polyamino-

10 methylene group via a nitrogen. The polyaminomethylenephosphonic acid moieties are preferably represented by Formula II, III or IV but not limited thereto. The polyaminomethyl-enephosphonic acid moieties preferably have a group present to permit coupling to a protein or electrophilic group(s) capable of being attached to protein(s) as described herein. The polyaminomethylenephosphonic acid moieties (represented by L-AP in Formula I) may be

15 linked to the protein (GF in Formula I) by a cleavable linker (represented by CL in Formula I), preferably an acid cleavable linker, as described herein.

The terms "growth factor" and "tissue growth promoting factor" mean any molecule which stimulates the proliferation, differentation, metabolism or migration of mammalian cells. The factors can be derived from natural sources or made by recombinant

20 DNA technology or chemical synthesis. Preferably the factors are purified.

The term "purified" as used herein refers to factors which, prior to mixing with the other growth factors, are 90% or greater, by weight, of the specified protein (i.e., is substantially free of other proteins, lipids, and carbohydrates with which it is naturally associated). A purified protein preparation will generally yield a single major band on a

25 polyacrylamide gel for each subunit. Most preferably, the purified factor used in the compositions of the invention is pure as judged by amino-terminal amino acid sequence analysis.

The ligands of this invention (AP of Formula I) may be in the form of their pharmaceutically acceptable salts. The term "ligand" as used herein is understood to include

30 these salts. The term "pharmaceutically acceptable salt" means a cation acceptable for pharmaceutical use. These are cations that are not substantially toxic at the dosage administered to achieve the desired effect. Illustratively, these salts include those of alkali metals, such as sodium and potassium; alkaline earth metals, such as calcium and magnesium; ammonium; light metals of Group IIIA including aluminum; and organic primary, secondary

35 and tertiary amines, such as trialkylamines, including triethylamine, procaine, dibenzylamine, N,N'-dibenzylethylenediamine, dihydroabiethylamine, N-(C_1-C_3)alkylpiperidine, and any other suitable amine. Sodium and potassium salts are preferred. The term "pharmaceutically acceptable" means suitable for administration to warmblooded animals, e.g. mammals,

-13-

especially human beings, and includes being nontoxic, e.g. suitable for pharmaceutical use and is not poisonous to the warm-blooded animal. The pharmaceutically acceptable salts of the compounds of the present invention are prepared by conventional ion exchange processes or by treating the ligand or compound of Formula I with an appropriate base.

5 Preparation of Polyaminomethylenephosphonic Acids

Various processes are known to prepare the polyaminomethylenephosphonic acid moieties of the present invention. A few of these processes are discussed below.

A. Preparation from Amines

Much literature exists which describes the preparation of
10 polyaminomethylenephosphonic acids, particularly the straight or branched-chain moieties, from amines. For example, US Patent 2,599,807, the disclosure of which is hereby incorporated by reference, teaches the preparation of polyaminomethylenephosphonic acids by heating an aqueous solution of an amine with chloromethylenephosphonic acid in the presence of a base such as sodium carbonate at pH > 10. Other examples are shown by the references given below
15 describing the preparation of ethylenediaminetetramethylenephosphonic acid (EDTMP) from the corresponding ethylenediamine (EDA), using a variety of phosphonomethylating agents as shown in the table below.

PREPARATION OF EDTMP

REACTION	REFERENCE
EDA + $ClCH_2PO_3H_2$	A.E. Martell et al, *Nature* 321 (1956); A.E. Martell et al, *S. Inorg. Nucl. Chem.* 33, 3353 (1971); A.E. Martell et al, *Inorg. Chem.* 15, 2303 (1976); A.E. Martell et al, *Inorg. Nucl. Chem. Letters* 7, 1103 (1971)
EDA + $ClCH_2PO_2H$, then Hg_2Cl_2	US Patent 3,160,632**
EDTA* + PCl_3 + H_3PO_4	US Patent 3,959,361**
EDA + H_3PO_3 + H_2CO	K. Moedritzer et al., *J. Org. Chem.* 31, 1603 (1966)
EDA + PCl_3 + H_2CO	British Patent 1,142,294**
EDA + (-OCH_2CH_2O-)P(O)Cl	US Patent 3,832,392**

*EDTA = ethylenediaminetetramethylenecarboxylic acid
35 ** = the disclosure of which is hereby incorporated by reference

Additional examples involving the preparation of cyclic polyaminomethylene-phosphonic acids from the corresponding amines can be found in US Patent 4,937,333, the disclosure of which is hereby incorporated by reference, and D. W. Swinkels et al., *Recl. Trav. Chim. Pays-BAS* 110, 124-128 (1991).

5   The preparation of polyaminomethylenephosphonic acids from amines via an intermediate aminomethylenephosphonate ester or mixed aminomethylenephosphonic acid ester is found in WO 91/07911, published June 13, 1991, the disclosure of which is hereby incorporated by reference. This process uses phosphonomethylating agents (e.g. formaldehyde and dialkyl phosphite) in an aqueous solution which forms the peralkyl phosphonate esters. These ester are then hydrolyzed to the aminomethylenephosphonic acids.

10 The reference also describes how to make alkyl or aryl substitutions on the carbon between the nitrogen and phosphorous by treatment of the ester with a strong base (e.g. n-butyl lithium) and alkylating with an alkyl or aryl halide.

    B. Preparation of Polyaminomethylenephosphonic Acids from Carboxylic Acids

15   Polyaminomethylenecarboxylic acids can be converted into the corresponding polyaminomethylenephosphonic acids by various known processes. The general reaction to transform the corresponding carboxylic acid involves a reagent capable of donating a phosphonic acid group. For example, the reaction of EDTA with PCl_3 in nitrobenzene to give EDTMP (e.g. US Patent 3,832,392, the disclosure of which is hereby incorporated by reference)

20   C. Preparation of Bifunctional Chelating Agents (BFCAs) having Amine Groups Capable of Conversion to Polyaminomethylenephosphonic Acids

    Simon et al.'s (assigned by unrecorded assignment to The Dow Chemical Company) copending US Patent Application Serial No. 565,379, filed August 9, 1990, the disclosure of which is hereby incorporated by reference, teaches the process to make various

25 linear or branched linear polyaminomethylenephosphonic acids that are capable of being attached to proteins. WO 84/03698, published September 27, 1984, describes open chain polyamine based bifunctional chelating agent intermediates which can be phosphonomethyl-ated to yield BFCAs. The synthesis of linear or branched polyalkylene polyphosphonate BFCAs is also described in US Patent 4,808,541, the disclosure of which is hereby incorporated by

30 reference.

    US Patents 3,994,966 and 4,622,420 and various references given therein describe how to make various ethylenediamine and diethylenetriamine based BFCAs with different groups for attachment to protein amine groups, all of which can be converted from the amine to a polyaminomethylenephosphonic acid BFC to be attached to the amine group of the

35 protein.

    Open chain polyaminomethylenephosphonic acid BFCAs containing the linkage through an aminocarboxylic acid can be prepared from the corresponding polyamine intermediate BFCAs as described in European Appln. 0 279 307, published August 24, 1988, the

-15-

disclosure of which is hereby incorporated by reference. US Patents 4,994,560, 5,006,643 and 5,064,956 also describe how to make various open chain and cyclic amine containing BFCAs which can be phosphonomethylated to yield the corresponding polyaminomethylenephosphonic acid BFCAs.

5   Cyclic polyamine BFCAs are taught in EP Appln. 0 353 450, published February 7, 1990, and describes how to prepare many different cyclic amine containing BFCAs which can then be carboxymethylated to yield aminomethylenecarboxylates. The amine containing intermediates of these BFCAs can alternatively be phosphonomethylated to yield the corresponding polyaminomethylenephosphonic acid BFCAs. Similarly, US Patent 4,885,363

10   describes a variety of 1,4,7,10-tetraazacyclododecane based amine containing intermediates which can be converted by phosphonomethylation to the corresponding polyaminomethylene-phosphonic acid BFCAs. The cyclic BFCAs based on the 1,4,7,10-tetraazacylododecyltetra-methylenephosphonic acid and the open chain BFCAs based on diethylenetriaminopenta-methylenephosphonic acid are a preferred group of polyaminomethylenephosphonic acid

15   BFCAs ligands.

The attachment group from the polyaminomethylenephosphonic acid BFCAs to the protein may be substituted from the cyclic polyaminomethylenephosphonic acid itself and are exemplified by J. P. L. Cox in *J. Chem. Soc., Chem. Commun.* 797 (1989), and M. K. Moi et al, in *J. Amer. Chem. Soc.* 110, 6266-6267 (1988). Similar compounds are shown in WO 89/11475,

20   published November 30, 1989.

Cyclic amine compounds based on the 1,4,8,11-tetraazacyclotetradecane have been described by M. K. Moi et al, in *Inorg. Chem.* 26, 3458-3463 (1987). These can be converted to the tetraaminomethylenephosphonic acid counterpart compounds by processes mentioned above. Similar compounds which may be used in the present invention are

25   described in US Patent 4,678,667. Additional examples of cyclic polyamines are described by T. J. McMurry et al. in *Bioconjugate Chem.* 3(2), 108-117 (1992). These intermediate bifunctional cyclic amines can be phosphonomethylated to give the corresponding polyaminomethylene-phosphonic acid BFCA (the L-AP portion of Formula I).

Bicyclopolyazamacrocylocarboxylic acid BFCA intermediates are disclosed in

30   Kiefer et al.'s (assigned by unrecorded assignment to The Dow Chemical Company) copending US Patent Application Serial No. 805,270, filed December 10, 1991, the disclosure of which is hereby incorporated by reference, can be phosphonomethylated to give the polyaminomethyl-enephosphonic acid BFCAs.

Bicyclopolyazamacrocylophosphonic acid BFCAs are disclosed in Kiefer et al.'s

35   (assigned by unrecorded assignment to The Dow Chemical Company) copending US Patent Application Serial No. 805,551, filed December 10, 1991, the disclosure of which is hereby incorporated by reference, teaches the process to make various bicyclopolyazamacrocylophos-phonic acid BFCAs having polyaminomethylenephosphonic acid BFCAs.

-16-

D. Preparation of Bifunctional Chelating Agents (BFCAs) having their Nitro Groups Reduced to Amine Groups

When the reduction of a nitro group, especially a nitrophenyl group, to the corresponding amino group is desired, particularly for compounds described in Paragraph C above, this reduction is readily accomplished by methods known in the art. For example, the processes listed in Survey of Organic Syntheses 1, 411-417 (1970), pub. John Wiley & Sons, and references contained therein.

E. Preparation of Bifunctional Chelating Agents (BFCAs) having their Amino Groups Converted to Electrophilic Groups

The BFCAs of the ligands of the present invention (e.g. L of Formula I) have a group present to permit coupling to a protein or electrophilic groups capable of being attached to a protein. The process to convert the amino group to electrophilic groups capable of being attached to a protein is well known in the art. Some references that provide suitable process are: C. F. Meares et al., *Acc. Chem. Res.* 17, 202-209 (1984) and references given therein; D. Parker, *Chem. Soc. Rev.* 19, 271-291 (1990) and references given therein; C. F. Meares et al., *J. of Protein Chem.* 2, 215-228 (1984) and references given therein; C. F. Meares "Protein Tailoring Food Med. Uses", Amer. Chem. Soc. Symp., 339-352 (1985), ed. R. E. Feeney and J. R. Whitaker, pub. Dekker, NY, NY. Examples of BFCAs have been given before. Additionally, many reagents are available for forming an amide bond in aqueous solution which brings together an amine containing molecule with a carboxylate containing molecule. For example, commercially available water soluble carbodiimides have been developed for this purpose [J. V. Staros, *Anal. Biochem.* 156, 220-222 (1986)].

Another method for linking two nucleophiles together is to convert one, such as an amine into a Michael acceptor, by reaction with acryloyl chloride or the chemical equivalent. This converts the amino group to the acrylamide group which can then react with a different nucleophilic amine (*Chem. Abst.* 83:80295b).

Other methods for preparing drug-carrier conjugations are described by M. J. Poznansky and R. L. Juliano in *Pharmacol. Rev.* 36, 278-336 (1984) and WO 90/14844.

F. Preparation of BFCAs by Other Methods

The molecules for attaching two nucleophilic moieties together are termed bifunctional crosslinking agents. If the two reactive ends of the molecule are the same, they are termed "homobifunctional" crosslinking agents. If the two reactive ends of the molecule are different, they are termed "heterobifunctional" crosslinking agents.

T. Kitagawa et al. in *Chem. Pharm. Bull.* 29, 1130-1135 (1981) and references given therein disclosed how to make heterobifunctional crosslinking agents for protein modification. Such reagents possess two selectively reactive groups such as a maleimide group (which reacts with thiol moieties) and N-hydroxysuccinimidyl ester (which reacts with amine groups such as lysine). These reagents allow the combination of two molecules together if one molecule

-17-

contains amine groups and the other molecule contains thiol group(s). Similar compounds containing a maleimide group has been reported by O. Nielsen in *Synthesis* 819-821 (1991).

Other bifuctional crosslinkers have been developed for the attachment of BFCAs or other electrophilic molecules to proteins or other nucleophillic substrates (US Patent

5   4,680,338 and references given therein); dialdehyde crosslinking agents [S. Avameas et al., *Scand. J. Immunol.* 8, 7-23 (1978)]; and commercially available crosslinking agents are shown in Pierce 1989 Handbook and General Catalog, pp 283-311 (Pierce, Rockford, IL).

Proteins and small molecules containing electrophilic groups such as amines (but also including other similar electrophilic groups) can be modified by reaction with

10 commercially available Traut's reagent [I. Wower, *Nuc. Acid Res.* 9, 4285-4291 (1981)] to convert amino groups to sulphydryl groups which can then react selectively with maleimide groups.

G. Cleavable Linkers

Sometimes it is advantageous that the linkage between the polyaminomethyl-enephosphonic acid BFCAs and the GF be reversible or cleavable under certain physiological

15 conditions. Such linkages are taught in the art and examples have been given above. Particularly advantageous are the linkers that are acid cleavable. Some examples of acid cleavable linkers are disclosed in US Patents 4,542,225 and 4,618,492 and the references mentioned therein. These cleavable linkers possess a cyclic anhydride at one end of the linker which reacts with amine groups, and a maleimido group at the other end of the linker which

20 reacts with sulphydryl groups. The linkage between the anhydride and amino groups (which forms an amide with a carboxylic acid hereby) is easily cleaved at acidic pH. Also US Patent 4,764,368 describes the use of cyclohexene-1,2-dicarboxylic acid anhydrides as a way to introduce acid cleavable amide functionality between a small molecule and a large molecule. WO 90/14844 describes the use of sugar derivatives as cleavable linkers in weakly acidic

25 conditions.

H. Presence of Metal Ions

Suprisingly, the addition of metal ions does not significantly inhibit the affinity of polyaminomethylenephosphonic acids for calcific surfaces. For example, the addition of calcium or samarium ions does not interfere with the affinity of 1,4,7,10-tetraazacyclodo-

30 decanetetramethylenephosphonic acid (DOTMP) for the calcific surface of hydroxyapitite.

For example, DOTMP has been shown to go to calcific sites *in vivo* when complexed with ions of samarium, holmium, gadolinium or yttrium in US Patent 4,976,950. Also ethylenediaminetetramethylenephosphonic acid (EDTMP) when complexed with calcium or other metal ions has also been shown to go to calcific sites *in vivo* (European Appln.

35 0 462 787, published December 27, 1991).

I. Clusters of Aminophosphonic Acids for Attachment to Proteins

Polymers having polyaminomethylenephosphonic acids, including dense star polymers such as described in European Appln. 0 272 180, published June 15, 1988, are

-18-

prepared by reacting the amine with formaldehyde and dialkyl phosphite in an aqueous
solution which forms the peralkyl phosphonate ester. This ester is then hydrolyzed to the
aminomethylenephosphonic acid on the dense star polymer. Particularly, the dense star is in
the PAMAM form having amine groups on its surface. These amine groups are then reacted

5   with phosphonomethylating agents to form the polyaminomethylenephosphonic acid, which
is then conjugated to the GF. The advantages of the dense star as the bone seeking moiety are
that it can be water soluble, have a controlled size, and specific groups and quantity of groups
available for conjugation to GF.

          The polymers of the L-AP of Formula I also include arborols (G. R. Newkome, *J.*

10 *Org. Chem.* 50, 2004-2006 (1985) which are capable of being attached to proteins, e.g. GF. The
arborols are monocascade spheres which possess a three-deminsional microenviroment having
the outer surface covered with polar functional groups. These arborols can be converted into
polyamines by methods known in the art for converting esters and alcohols into amines (e.g.
Survey of Organic Synthesis 1, 411-417 (1970), pub. John Wiley and references given therein).

15 The arborol polyamines can be converted into polyaminomethylenephosphonic acids by the
methods described above. By starting the arborol cascade polymer with the appropriate group
such as *p*-nitrobenzyl bromide, the arborols can contain a group capable of attaching to
proteins. Thus aborols containing polyaminomethylenephosphonic acid groups and a group
capable of attaching to a protein by either a stable covalent linkage or cleavable linkage can be

20 prepared.

          Also, other polymers, either linear or branched, containing amines and a group
capable of attachment to a protein have been described [V. P. Torchilin et al., *Byull. Eksp. Biol.
Med.* 102, 63-65 (1986)]. Such polyamine containing polymers can be converted by methods
described above to polymers containing polyaminomethylenephosphonic acid groups.

25   By adjusting the stoichiometries of the protein to reactive group containing
cluster of polyaminomethylenephosphonic acid, the type of product can be altered. Also, by
adjusting the stoichiometry during the step that introduces the linkage containing the reactive
group, a cluster compound can be linked to two molecules of protein which may be identical to
each other or different molecules of protein.

30   J. Alternate Processess

          Selective attachment of the polyaminomethylenephosphonic acid BFCAs to
noncritical amine groups on a GF may be accomplished by first mixing the GF with a soluble or
solid support immobilized form of the receptor from cells which will form a GF-receptor
aggregate. This aggregate can then be reacted with an excess of the reactive form of the poly-

35 aminomethylenephosphonic acid BFCA. Since the GF is bound to the receptor, only amine
groups not involved in the GF receptor interaction will be modified. The GF modified in this
fashion is th n able to interact with its receptor *in vivo* to yield biological activity. The
aggregate between the modified GF and the receptor can then be broken down into the now

-19-

selectively modified GF and the reusable receptor, which after separation will yield fully active modified GF that still recognizes the receptor.

The compounds of Formula I can be prepared in several different ways. For example, the AP portion can be reacted with L to form the L-AP portion; the L-AP portion then

5   attached to the CL; with the CL-L-AP group then attached to the GF. Alternatively, GF is reacted with CL to form the GF-CL portion; which GF-CL is then reacted with L-AP to form compounds of Formula I. The degree of modification of the GF is described by the q term of Formula I, which is arrived at experimentially by adjusting the concentrations, time, temperature, pH, and stoichiometry of the reactions between GF, CL and the L-AP molecules.

10 Formulations

The formulations of the present invention are in the solid or liquid form. These formulations may be supplied as a single substance for direct use or as two or more substances (e.g. in kit form) such that the two components are mixed at the appropriate time prior to use. Whether premixed or as a kit, the formulations may require a pharmaceutically-acceptable

15   carrier or adjuvant.

The compounds of this invention may also be administered parenterally, that is, subcutaneously, intravenously, intramuscularly, or interperitoneally, as injectable dosages of the compound in a physiologically acceptable diluent with a pharmaceutical carrier which can be a sterile liquid or mixture of liquids such as water, saline, aqueous dextrose and related

20   sugar solutions, an alcohol such as ethanol, isopropanol, or hexadecyl alcohol, glycols such as propylene glycol or polyethylene glycol, glycerol ketals such as 2,2-dimethyl-1,3-dioxolane-4-methanol, ethers such as poly(ethyleneglycol) 400, an oil, a fatty acid, a fatty acid ester or glyceride, or an acetylated fatty acid glyceride with or without the addition of a pharmaceutically acceptable surfactant such as a soap or a detergent, suspending agent such as

25   pectin, carbomers, methylcellulose, hydroxypropylmethylcellulose, or carboxymethylcellulose, or emulsifying agent and other pharmaceutical adjuvants.

Illustrative of oils which can be used in the parenteral formulations of this invention are those of petroleum, animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, sesame oil, cottonseed oil, corn oil, olive oil, petrolatum, and mineral oil.

30 Suitable fatty acids include oleic acid, stearic acid, and isostearic acid. Suitable fatty acid esters are, for example, ethyl oleate and isopropyl myristate. Suitable soaps include fatty alkali metal, ammonium, and triethanolamine salts and suitable detergents include cationic detergents, for example, dimethyl dialkyl ammonium halides, alkyl pyridinium halides, and alkylamines acetates; anionic detergents, for example, alkyl, aryl, and olefin sulfonates, alkyl, olefin, ether,

35   and monoglyceride sulfates, and sulfosuccinates; nonionic detergents, for example, fatty amine oxides, fatty acid alkanolamides, and polyoxyethylenepoly-propylene copolymers; and amphoteric detergents, for example, alkyl-beta-aminopropionates, and 2-alkyl-imidazoline

-20-

quarternary ammonium salts, as well as mixtures. The parenteral compositions of this invention will typically contain from about 0.001% to about 10% by weight of compound of Formula I in solution. Preservatives and buffers may also be used advan-tageously. In order to minimize or eliminate irritation at the site of injection, such compositions may contain a non-

5 ionic surfactant having a hydrophile-lipophile balance (HLB) of from about 12 to about 17. The surfactant can be a single component having the above HLB or can be a mixture of two or more components having the desired HLB. Illustrative of surfactants used in parenteral formulations are the class of polyethylene sorbitan fatty acid esters, for example, sorbitan monooleate.

Other possible formulations for the compounds of Formula I include: orally, using

10 known oral pharmaceutical formulations some of which may contain magnesium hydroxide in excessive amounts [P. J.Neuvonen et al., *Eur. J. Clin. Pharmacol.* 35, 495-501 (1988)]; trans-dermal delivery using known procedures from B. Kari, *Diabetes* 35, 217 (1986), B. R. Meyer et al., *Clin. Pharmacol. Ther.* 44, 607 (1988); implants using adsorption of the compound onto activated carbon particles which are then implanted or injected [A. Hagiware, *Gan to Kagaku*

15 *Ryoho* 15, 1038-1042 (1988) or *Chem. Abst.* 109:156158n (1988)]; and nasal delivery, either alone or in combination with permeation inhancers [W. A. Lee, *BioPharm.* 22-25 (Nov/Dec, 1990) or *Wall Street J.* B1, (August 16, 1989)]; and others by R. Langer, *Sci.* 249, 1527-1533 (Sept. 28, 1990). The formulations may also be applied locally to the site of the injured or depleted bone by direct topical application to that site. The latter method may require surgery to expose

20 the site of injured or depleted bone.

Bone regeneration using the compounds of the present invention is more effective than that achieved in the absence of treatment (i.e. without applying exogenous agents) or by treatment with similar levels of unmodified growth promoting factors, i.e. only GF of Formula I. Because of the relatively high cost of GF production and the potential ability

25 of GF to cause adverse toxic affects when delivered in high doses systemically, it is also desireable to use the ligand to ensure delivery to the desired site and reduce the overall dose of GF and its toxic effects to the mammal.

In the method of regenerating bone of a mammal, especially a human patient, according to the invention there is administered to the mammal either by direct application to

30 the area of injured or depleted bone or by indirect application, for example, via systemic circulation (such as following parenteral, intramuscular or subcutaneous injection) an effective amount of a composition that includes a compound of Formula I.

The amount of $(CL)_2$-L-AP of Formula I which is needed to aid in bone delivery of the GF can be any effective amount. The amount of compound of Formula I to be administered

35 in order to treat any of th diseases desired for such a delivery system can vary widely according to the particular dosage unit employed, the period of treatment, the age and sex of the patient treated, the nature and extent of the disorder treated, and other factors well-known to those

-21-

practicing the medical arts. Moreover the compounds of Formula I can be used in conjunction with other agents known to be useful in the treatment of bone diseases.

The effective amount of compounds of Formula I to be administered according to the present invention will generally range from about 0.005 to 50 mg/kg of body weight of the

5 patient and can be administered as frequently as one or more times per day. The compounds of Formula I can be administered in a pharmaceutically acceptable formulation as described above. The compounds of Formula I can have one or more different active compounds administered either simultaneously or sequentially; and such compounds may be administered with other known active agents for regenerating bone.

10 The invention will be further clarified by a consideration of the following examples, which are intended to be purely exemplary of the present invention.

Definitions

The terms used in the present examples and not defined previously herein are as follows:

15   BMP = bone morphogenetic proteins

BSA = bolvine serum albumin

FCS = fetal calf sera

HPLC = high pressure liquid chromatography

HSA = human serum albumin

20   PBS = phosphate buffered saline

RP-HPLC = reverse phase HPLC

SCN-BDTMP = 4-isothiocyanatobenzyldiethylenetriaminopentamethylenephos-
phonic acid

All solvents and reagents were obtained from commercial suppliers and used

25 without further purification, except as defined below.

All water was passed through a Barnstead NANOpure™ ion exchange/carbon bed water purification system and had a resistivity of about 18.5 megaohms

Acetic acid was glacial acetic acid from Aldrich Chemical Co. at greater than 99.99%

30   1% HSA in 1.0 mL aliquots in PBS (20mM phosphate, pH 7.4, 0.15M NaCl) were received frozen from the Institute of Molecular Biology, Inc. (IMB)

^{125}I-PDGF was obtained from IMB (frozen in 135 µL aliquots, containing 100 µg of nonradioactive IGF and 26.5 µCi of carrier free ^{125}I-PDGF), as well as unlabelled purified recombinant PDGF-BB and IGF-I, all in 0.1M acetic acid

35   Centricon™ membrane filters of 10,000 and 3,000 molecular weight cutoff (Amicon Division of W. R. Grace and Co.) were used for washing and concentrating the aqueous protein solutions

-22-

Growth Factor Modification

General Methods

   Purified recombinant human PDGF-BB and recombinant human IGF-I and their respective radioiodinated forms were supplied by the Institute of Molecular Biology, Inc. The identities of both PDGF-β and IGF-I were established by amino acid composition and amine terminal amino acid sequence. Amino acid composition was determined by gas phase hydrolysis of the protein followed by RPHPLC essentially according to the method of Eveleigh and Winter [J. W. Eveleigh and G. D. Winter, "Amino Acid Composition Determination" Protein Sequence Determination, pp. 91-95 (1970)]. The amine terminal amino acid sequence was obtained by the Edman degradation followed by formulation and analysis of the PTH amino acid by HPLC essentially according to the method of Hunkapiller and Hood [M. W. Hunkapiller and L. E. Hood, *Methods in Enzymology* 91, 486-489, (1983)]. Purities were determined using scanning densitometry of Coomassie blue and silver stained SDS PAGE gels and RP-HPLC.

STARTING MATERIALS

Example A

Preparation of PDGF-BB and IGF-I

   Both PDGF-BB and IGF-I were produced by standard recombinant DNA techniques and purified by conventional chromatography. Both process are well known to those skill in the art. (PDGF: US Patents 4,061,757 and 5,045,633; IGF-I: Y. Sato et al., *J. Biochem.* 101, 1247-1252 (1987) and Wong et al., *Gene* 68, 193-203 (1988).

   Analysis of the amine terminal amino acid residues 1 through 20 of the PDGF-BB (provided by IMB) gave a single, intact amine terminus and was identical to those residues predicted by the cDNA sequence.

   Sequence analysis of the amine terminal residues 1 through 53 of the human IGF-I (produced by IMB) yielded a single intact amine terminus identical to those residues predicted by the cDNA sequence. The amino acid compositions were found to be consistent with that predicted by the integrated human IGF-I gene. Both growth factors were greater than 95% pure.

Example B

Biological Activity of PDGF-BB and IGF-I

   Potency or bioactivity of each factor was measured by cell culture mitogenic assay in which the effective dose at half-maximal stimulation (ED_{50}) is defined as one unit. Both bioassays are based on the measurement of the incorporation of [3H]-Thymidine into DNA of BALB/c 3T3 mouse fibroblasts. The 3T3 cells are seeded, in DMEM supplemented with 10% FCS, into 96 well assay plates at 2500 cells per well. Assay plates are incubated (37°C) for seven days prior to use. This allows for the depletion of serum components and induces quiescence in the cells. In the PDGF-BB bioassay standards and test samples are added in triplicate on the afternoon of the seventh day and are allowed to incubate overnight for 18 hours. The cells are

-23-

then exposed to 1.0 μCi of ^{3}H-thymidine for 6 hours, which is incorporated according to the biological activity of the PDGF.

In the bioassay for IGF-I, the cells are preincubated with PDGF-BB, prior to the addition of test samples and standards. The preincubation enables the cells to respond to IGF-I

5 when the IGF-I is added in the presence of Epidermal Growth Factor (EGF). After an overnight incubation period the cells are exposed to 1.0 μCi of ^{3}H-thymidine which is incorporated according to the biological activity of IGF-I.

In both the PDGF-BB and IGF-I assays, following extensive washing, the cells are lysed and the samples are quantitated on a liquid scintillation counter. The resulting data are

10 plotted to generate a dose response curve from which unit activity determinations can be made.

A unit is defined that concentration of GF which induces 50% of the maximum cellular response in the appropriate assay (ED_{50} value). Maximum response in the PDGF asnd IGF-I assays is defined as the cellular response to a 5% Fetal Calf Sera (FCS) standard. The

15 specific activity was calculated by dividing the number of units based on the mitogenic assay by the mass as determined by amino acid analysis. The potency for both PDGF-BB and IGF-I prior to protein modification was 1 to 3 ng/mL (see Figure 3 for PDGF-BB; Figure 4 for IGF-I). Typically, the specific activities were in the range of 3 to 10 x 10^5 units per mg of protein.

Example C

20 Radiolabelled ^{125}I-PDGF

The "cold" PDGF described in Example A was labelled by the [^{125}I]-Bolton Hunter method which principally labels the primary amines of accessible lysine and N-terminal residues. ^{125}I-PDGF is also commercially available from NEN. NEN normally supplies the ^{125}I-PDGF in a sodium citrate buffered salt solution containing 1% BSA as a carrier protein. Prior to

25 use this BSA must be removed from the commercially available ^{125}I-PDGF to allow efficient modification of the PDGF. In order to produce the carrier free form of ^{125}I-PDGF, antibody affinity chromatography was used. The immobilized anti-PDGF antibody (supplied by IMB) exhibits specific binding to the AB and BB PDGF isoforms, and no binding to the AA homodimer. 42.6 μCi ^{125}I-PDGF in a neutral pH buffer was applied to a 200 μL anti-PDGF

30 column. The BSA was exhaustively flushed from the column with PBS and the ^{125}I-PDGF eluted in 0.1M acetic acid. Based on a specific activity of 35 μCi/μg this particular lot of ^{125}I-PDGF is estimated to have, on average, one [^{125}I] attached for every 1.8 PDGF dimers.

Example D

Radiolabelled ^{125}I-IGF

35 The "cold" IGF described above was labelled with [^{125}I] using the lactoperoxidase or chloramine T method which iodinates tyrosine residues forming iodotyrosyl products. ^{125}I-IGF-I is commercially available from NEN. ^{125}I-IGF-I was supplied carrier free and freeze dried

-24-

from 100 uL of 100mM sodium citrate, pH 4.5. Based on a specific activity of 208 µCi/µg, this lot of [125I]-IGF-I is estimated to have on average, one [125I] attached for every 1.4 IGF-I protein chains.

Example E

5   Preparation of two PDGF solutions

A vial containing radioactive PDGF was placed in the well of a Capintec dose calibrator which was adjusted for [125I]. A vial containing 50 µg of PDGF was rinsed with 200 µL of 0.1M acetic acid and added to a vial containing 100 µg of cold IGF and [125I]-PDGF in 135 µL of 0.1M acetic acid. The cold PDGF was vial was rinsed with 7 more 200 µL portions of 0.1M acetic

10   acid, which rinses were combined to provide one sample containing radioactive and nonradioactive PDGF and nonradioactive IGF. The combined sample vial was shaken to mix and two 750 µL aliquots of the solution were removed. Each aliquot was placed on a separate membrane of a centricon 10, labelled 1 and 2. Appropriate volume and gamma readings were taken which verified the volume and radioactivity before and after dividing of the sample. The

15   two aliquot samples were spun in a centrifuge and placed in new tared filtrate cups.

Each membrane filter (1 and 2) was then treated with 1.0 mL of 3.0M sodium bicarbonate buffer and recentrifuged. The two cups were removed and replaced with new tared filtrate cups. Both membrane retained samples read 10.5 µCi. The two membrane filters were then treated with an additional 1.0 mL of 3.0M sodium bicarbonate buffer and

20   recentrifuged. Both filtrate cups were removed and replaced with new tared filtrate cups. Each of the two samples read 10.5 µCi on the membrane. The washing procedure described above was done to remove the carrier nonradioactive IGF which should come through the membrane. The remaining material on the membrane was then ready for conjugation.

Example F

25   Preparation of H$_2$N-BDO3TMP

The free base, 1-(α-carboxyl-2-methoxy-5-nitrobenzyl)-1,4,7,10-tetraazacyclododecane, 513 g (1.3 mmol) was added to a stirred slurry of 776 mg (4.7 mmol) of triethyl phosphite and 141 mg (4.7 mmol) of paraformaldehyde. The resulting slurry was heated with stirring to 95°C for 2 hours, cooled to room temperature and concentrated *in*

30   *vacuo* to give a viscous oil. The oil was chromatographed using a basic alumina column and eluted with chloroform and, after evaporation of the solvent, to yield (55%), as a light yellow viscous oil, of the ester, 1-(α-carboxyl-2-methoxy-5-nitrobenzyl)-1,4,7,10-tetraazacyclo-dodecane-4,7,10-trimethylenephosphonic acid hexaethyl ester.

The ester, 250 mg (0.3 mmol) was hydrolyzed by stirring with 3 mL of

35   concentrated HCl at 100°C for 18 hours. The aqueous solution was freeze-dried to give (80%), as a cream colored solid, 1-(α-carboxyl-2-methoxy-5-nitrobenzyl)-1,4,7,10-tetraazacyclodo-decane-4,7,10-trimethylenephosphonic acid (O$_2$N-BDO3TMP).

-25-

A 100 mg portion of O₂N-BDO3TMP was dissolved in 20 mL of water. After
purging the system with nitrogen, 120 mg of 10% Pd/C was added and the suspension placed
under an atmosphere of hydrogen with constant vigerous stirring. After 3 hours the catalyst
was removed via filtration and the filtrate lyphilized to give 89.1 mg of H₂N-BDO3TMP as a
5   chocolate colored solid. A 23.4mM solution of this ligand was prepared by dissolving 89.1 mg
of H₂N-BDO3TMP in 4.26 mL of water.

Example G
Preparation of SCN-BDTMP

        (1.) A 100 mL three necked, flask was loaded with 2.0 g (5.76 mmol) of 1-(4-
10   nitrobenzyl)diethylenetriamine trihydrochloride [prepared by the procedure described by
M. W. Brechbiel et al. in *Inorg. Chem.* 25, 2772-2781 (1986)]. A separate solution of 10.93 g
(0.108 mol) of concentrated HCl and 6.91 g (0.086 mol) of phosphorous acid was prepared and
added to the reaction flask. The flask was fited with a therometer, reflux condenser and a stir
bar. With constant stirring the reaction solution was brought to reflux. An addition funnel was
15   loaded with 12.0 g (0.144 mol) of 37% by wt formaldehyde solution, attached to the reaction
flask, and dripped into the heated mixture at a rate of about 1 mL/min. The reaction was held
at reflux for an additional 16 hours, then reduced in volume under vacuum to produce an
amber semisolid. The solid was taken up in about 2 mL of water and added dropwise with
vigorous stirring to about 800 mL of methanol. The resulting white percipitate was removed by
20   filtration and dried at 45°C to yield, as an off white crude solid, 2.32 g (57%) of 1-(4-
nitrobenzyl)diethylenetriaminepentamethylenephosphonic acid (O₂N-BDTMP).

        (2.) A 500 mg portion of crude 1-(4-nitrobenzyl)diethylenetriaminepenta-
methylenephosphonic acid was purified on a 1.5 cm Q-Sepharose (Pharmacia) anion exchange
column eluting with 0 to 1M ammonium acetate gredient. The desired product, 1-(4-nitro-
25   benzyl)diethylenetriaminepentamethylenephosphonic acid, is the most retained peak at UV
280 nm. Collection of the last eluting peak resulted in about 43 mL of solution containing
purified product. The volume was reduced to about 26 mL and treated with 200 mg of 10%
Pd/C. The suspension was put under a balloon of hydrogen gas as stirred vigerously for about
2.5 hours. HPLC (anion exchange, eluting with 0 to 1M ammonium acetate over 30 minute
30   gradient a 2 mL/min) indicated a slight shift to a shorter retention time for the reduced product.
The suspension was then filtered, and the filtrate lyophilized to give 259 mg of white glassy
solid. HPLC indicated a purity of about 92%. The product was further purified by preparative
anion exchange chromatrography as described above to give, as a white solid, 144 mg of the
ammonium salt of 1-(4-aminobenzyl)diethylenetriaminepentamethylenephosphonic acid, with
35   a purity by anion exchange HPLC of >95%. The product was further characterized by as
follows.

Decoupled P-31 NMR showed 3 singlets in the expected 2:2:1 ratio.

^{1}H NMR(D$_2$O)

δ 2.59-3.67 (m, 17H), 4.13 (b. 2H), 6.91 (d, 2H), 7.21 (d, 2H).

5   ^{13}C NMR(D$_2$O)

δ 52.0, 52.6, 53.3, 53.4, 54.1, 54.9, 55.8, 57.1, 57.1, 58.3, 58.3, 65.2, 120.5, 131.1, 133.5, 146.8.

(3a.) A 0.09M solution of the ammonium salt of 1-(4-aminobenzyl)diethylenetri-
aminepentamethylenephosphonic acid in water was prepared by dissolving 146 mg of the
phosphonic acid in 1.913 mL of water. A 328 μL portion of this solution (containing about 25

10   mg of the phosphonic acid) was placed in a 10 mL vial containing 1 mL of water and 1 mL of
chloroform. To the emulsion that formed was added 100 μL of thiophosgene with vigerous
stirring for about 1 hour. The chloroform layer was removed and the aqueous portion
extracted with water using four 1 mL portions of chloroform. The washed aqueous solution of
the product was frozen in a dry ice/acetone bath and lyophilized to give 26.5 mg of puffy white

15   powder. A 0.5 mg portion was dissolved in 200 μL of water. A 6 μL portion of this solution was
diluted with 3.8 mL of water and the UV spectrum obtained showing absorption maxima at 270
and 283 nm confirming the product, 1-(4-isothiocyanatobenzyl)diethylenetriaminepenta-
methylenephosphonic acid (SCN-BDTMP).

(3b.) A 43 mg (51 μM) portion of 1-(4-aminobenzyl)diethylenetriaminepenta-

20   methylenephosphonic acid was dissolved in 2 mL of water and mixed with 1 mL of chloroform.
To the stirred solution was added 150 μL of thiophosgene in one portion. After vigerous
stirring for 2.5 hours the chloroform layer was removed and the aqueous portion extracted
with water using three 3 mL portions of chloroform. The washed aqueous solution of the
product was frozen in a dry ice/acetone bath and lyophilized to give 45.4 mg of puffy white

25   powder. A 2.4 mg portion was dissolved in 960 μL of water. A 6 μL portion of this solution was
diluted with 3.8 mL of water and the UV spectrum obtained showing peaks at 272 and 282 nm
confirming the product, 1-(4-isothiocyanatobenzyl)diethylenetriaminepentamethylene-
phosphonic acid.

(3c.) Preparation of 3.47mM and 34.7mM solutions of SCN-BDTMP

30   A 100 mg portion (1.389 x 10^{-5} mol) of SCN-BDTMP was dissolved in 400 μL of 0.3M
sodium bicarbonate buffer. The resulting solution had a pH of 7.51 which was adjusted to a pH
of 9.48 by the addition of 5 μL of 50% (wt) sodium hydroxide. The final solution was 34.7mM
of SCN-BDTMP.

A 5 μL portion of the above prepared 34.7mM SCNBDTMP solution was added to

35   45 μL of 0.1M sodium bicarbonate and mixed thoroughly. The final solution was 3.47mM of
SCN-BDTMP.

-27-

Example H

Preparation of four IGF Solutions

        Vials of 100 µL of lyophilized radiolabelled ^{125}I-IGF-I (from NEN) or lyophilized recombinant IGF-I (from IMB) were each reconstituted with 100 µL of water to give solutions of

5   1 mg/mL of IGF containing 0.5M of sodium chloride in 20mM of Tris-hydrochloric acid with 0.25% BSA as a carrier protein. The recombinant IGF vial's contents were dissolved in 10mM of acetic acid and transferred into the glass serum vial containing iodinated IGF dissolved in 535 mg of 10mM acetic acid. This combined solution of IGF containing tracer ^{125}I-IGF-I was dialysed (Spectra/Por7™ membrane from Spectrum Medical Industries) against three 1 L portions of

10   10mM acetic acid. The contents of the dialysis bag were then divided into 4 equal volumes and placed onto the membranes of 4 separate Centricon™ microconcentrator membrane filters (3000 molecular weight cutoff). The Centricons™ were then spun to concentrate the solutions and the contents of the membrane washed with one 1.6 mL of 0.3M sodium bicarbonate buffer at pH = 9.48. This method gave 4 Centricon™ membrane units containing the amounts of IGF

15   shown below:

IGF

Centricon™ No.	IGF on membrane µg*
1	41.2
2	41.7
3	45.7
4	47.7

* approximate

25   Example I

Preparation of two IGF solutions

        Frozen radiolabelled ^{125}I-IGF as a 150 µL aliqout containing 300 µg of IGF-I and 23.6 µCi of carrier free ^{125}I-IGF in 0.1M acetic acid was allowed to thaw and then diluted to a final volume of about 1.5 mL using 0.1M acetic acid. A 700 µL aliqout of this solution was

30   placed on each of 2 Centricon™ microconcentrator membrane filters (3000 molecular weight cuttoff). The Centricons™ were then spun to concentrate the solutions and the contents of the membrane washed with two consecutive 1 mL portions of 0.3M sodium bicarbonate buffer at pH = 9.48. The calculated amount of IGF (with tracer ^{125}I-IGF) in membrane no. 1 was 130 µg (1.7 x 10-8 mol) and in membrane no. 2 was 127 µg.

35

Example J

Preparation of 1,3-propanediamine-N-(carboxy-propyl)-N,N',N'-trimethylene-phosphonic acid
(PDTMP)

The structure of the compound PDTMP is shown by the following formula:

5

$$HOOC \longrightarrow (CH_2)_3 \diagdown N \longrightarrow CH_2 \longrightarrow CH_2 \longrightarrow CH_2 \longrightarrow N \diagup^{CH_2-PO_3H_2}_{CH_2-PO_3H_2}$$

$$H_2O_3P-CH_2$$

10

Commercial grade 3-aminopropyl-2-pyrrolidinone (5.0 g) was added slowly to a
solution of phosphorous acid (9.7 g) in 6M hydrochloric acid (50 mL). The yellow solution was
heated to reflux and treated dropwise with formalin (9.23 mL of 37% solution) over at two

15 hour period. After refluxing an additional 18 hours, the solution was cooled and the solvent
removed to give a viscous oil. Dripping the oil into 200 mL of methanol gave 5.15 g of PDTMP
after filtering and drying. This compound showed the expected two singlets in the decoupled
^{31}P-NMR spectrum at 8.2 ppm and 7.5 ppm (relative to H_3PO_4) in a 2:1 ratio.

This compound may be used as a ligand AP in Formula I where the L is through the

20 COOH group.

Example K

Preparation of Ethylenediamine-N-(4-amino-phenethyl)-N,N',N'-trimethylenephosphonic Acid
(APEDTMP)

The structure of the compound APEDTMP is shown in the following formula:

25

$$H_2N-\bigcirc-CH_2CH_2 \diagdown N \longrightarrow CH_2 \longrightarrow CH_2 \longrightarrow N \diagup^{CH_2-PO_3H_2}_{CH_2-PO_3H_2}$$

$$H_2O_3P-CH_2$$

30

Three 250 mL Erlenmeyer flasks were loaded with 120 mL of ethylenediamine
(EDA) and equipped with a stirring bar and placed on a stirrer. Three 10 g samples of p-

35 nitrophenethylbromide were weighed and added slowly to the stirring flasks containing EDA
over a thirty minute period. Following the addition of p-nitrophenethyl bromide the flasks
were allowed to stir overnight (sixteen hours) at room temperature. The contents of the flasks

-29-

were transferred to a 500 mL round bottomed flask and attached to a simple distillation apparatus. The excess EDA was distilled from the desired product at a temperature of 28 to 32°C under vacuum. Following the removal of most of the starting EDA the viscous oil was dissolved in water and extracted three times with 75 mL of methylene chloride (CH_2Cl_2). The

5   CH_2Cl_2 layers were combined and concentrated in a rotoevaporator to yield 29.34 grams of a viscous dark oily liquid.

All of this sample was added to a 500 mL round bottomed flask along with 100 mL of 1.5M HCl. The aqueous layer was treated with activated carbon heated to 90°C, then filtered through a paper filter. The aqueous layer was stripped under vaccum and dried in a vacuum

10   dessicator overnight.

The resulting dry solid was washed with cold methanol and filtered to give 15.60 g of N-(p-nitro-phenethyl)ethylenediamine as the hydrochloride salt. An additional 6.77 g of product was obtained by evaporating the filtrate and washing with methanol to give a combined yield of 72%.

15   A 250 mL three-necked, round bottomed flask was loaded with 20.0 g (0.063 moles) of N-(p-nitro-phenethyl)ethylenediamine trihydrochloride salt. To this flask was added 9.0 g of deionized water and 22.79 g (0.219 moles) of concentrated hydrochloric acid in a solution with 17.16 g (0.209 moles) of phosphorous acid.

The flask was attached to a reflux condenser, equipped with a stir bar, and set on

20   a stirrer. A 10 mL syringe was filled with 17.74 g (0.219 moles) of 37% formaldehyde solution and attached to a syringe pump pre-calibrated to deliver at a flow rate of 0.1 mL/min. The reaction solution was brought up to reflux temperature, then with constant stirring, the formaldehyde was slowly added to the flask over a three hour period.

Following the addition of the formaldehyde solution, the reaction was allowed to

25   reflux and stir for an additional three hours. The reaction was allowed to cool and the water was removed under vaccum to give a viscous, dark solid. This was slowly added to methanol resulting in a light brown solid. This precipitate was filtered and dried to give 20 g (65% yield) of N-(p-nitrophenethyl)-ethylenediamine-N,N',N'-trimethylene phosphonic acid. A 10 g sample of this triphosphonic acid was dissolved in 20 mL of water and neutralized with 2 mL of

30   concentrated ammonium hydroxide. This crude ammonium salt was then purified using reverse phase preparative HPLC eluting with water. A sample of N-(p-nitrophenethyl)ethylenediamine-N,N',N'-trimethylene phosphonic acid (710 mg, 1.21 μmoles) purified in this manner was dissolved in 50 mL of water and placed in a hydrogenation bottle with a 235 mL volume capacity. A blanket of nitrogen was gently blown into the bottle to

35   displace the air. Approximately 50 mg of 10% Pd on carbon was added as a catalyst and any catalyst on the vessel sides was rinsed down with water. Once all the catalyst was under water, the nitrogen padded bottle was placed on a Parr hydrogenation shaker apparatus. After removal of the nitrogen by means of a vacuum line, an atmosphere of hydrogen was

-30-

introduced. This flushing was repeated to ensure that all oxygen had been removed. The final charge of hydrogen gave a pressure of 35 psi. The hydrogenation was started by shaking the bottle and continued until the hydrogen uptake ceased (2.5 hours). This pressure drop and the known volume of hydrogen can be used to calculate the moles of hydrogen used which in this

5   case was 99.2% of theoretical. The reaction mixture was then carefully vacuum filtered (after removal from the depressurized hydrogenation apparatus) through a glass frit and the catalyst thus separated was washed with four 25 mL portions of water. The combined aqueous filtrate was then frozen and lyophilized overnight to yield 580 mg (86% yield) of N-(4-aminophenyl)-ethylethylenediamine-N,N',N'-trimethylenephosphonic acid as the ammonium salt (APEDTMP).
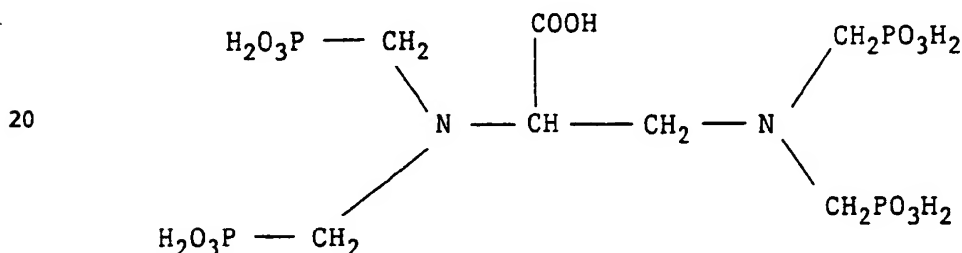
10   The decoupled ^{31}P-NMR Spectrum of this compound showed a singlet at 8.0 ppm (relative to H_3PO_4) and a singlet at 16.9 ppm in the expected ratio of 1:2, respectively.

            This compound may be used as a ligand AP in Formula I where the L is through the NH$_2$ group.

Example L

15   Preparation of 1-(Carboxy)ethylenediaminetetramethylenephosphonic Acid (CEDTMP)

            The structure of the compound CEDTMP is shown below:

20

$$H_2O_3P - CH_2 \diagdown$$
$$\qquad\qquad\qquad N - CH - CH_2 - N$$
$$H_2O_3P - CH_2 \diagup$$

with COOH on the CH, and CH$_2$PO$_3$H$_2$ and CH$_2$PO$_3$H$_2$ on the N.

25   To a solution of phosphorous acid (12.9 g) in 6M hydrochloric acid (50 mL) was added 5.0 g of 2,3-diaminoproprionic acid dihydrochloride (Aldrich Chemical Co., Milwaukee, WI). The solution was heated to reflux and treated dropwise with formalin (37% formaldehyde in water, 12.3 mL) over a period of 45 minutes. After an additional eighteen hours of reflux, the solvent was removed and the oil dissolved in methanol (20 mL). This solution was then

30   added with stirring to absolute ethanol (200 mL) to yield a fine precipitate. This solid was then filtered and dried, yielding 7.37 g of CEDTMP. An aliquot of this solution was diluted ten-fold and analyzed by high performance liquid chromatography (HPLC). HPLC was performed on a Dionex™ 2010i Ion Supression Chromatography System (Sunnyvale, CA) using an AS-7 anion exchange column (8mm x 25cm) and eluting with 0.03M nitric acid at a flow rate of 0.6 mL/min

35   with ultraviolet detection monitored at 330 nanometers. This compound had a retention time of 10.11 minutes.

This compound may be used as a ligand AP in Formula I where the L is through the COOH group.

Example M

Preparation of 1-(4-aminobenzyl)ethlene-diaminetetramethylenephosphonic acid (ABEDTMP)

The structure of the compound ABEDTMP is shown in the formula:
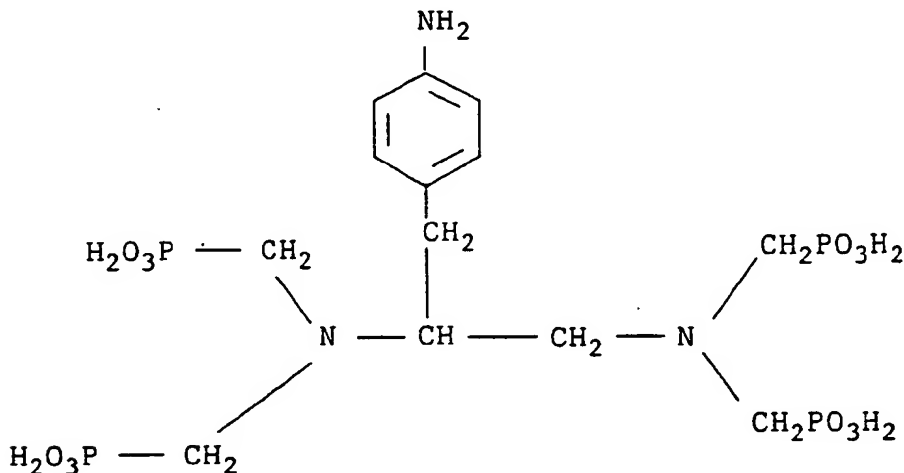
To a 50 mL round bottomed flask was added 0.6 g (0.2256 moles) of 1-(4-nitrobenzyl)ethylenediamine, concentrated HCl (1.24 g, 0.0118 mole) and then phosphorous acid (0.0925 g, .0113 mole). The solution was brought to reflux and then treated with 0.956 g (0.0118 mole) of 30% formaldehyde dropwise over a ninety minute period. The reaction was then kept at reflux overnight, cooled to room temperature and then added dropwise to 100 mL of cold methanol. The resulting precipitate was filtered and dried to yield 1-(4-nitrobenzyl)-ethylenediaminetetramethylenephosphonic acid, which was dissolved in 60 mL of water in a hydrogenation flask. A 50 mg sample of 5% Pd on carbon was added and the flask connected to a Parr hydrogenation apparatus and shaken until hydrogen uptake ceased. The solution was then filtered to remove the catalyst and the resulting clear filtrate was frozen and freeze-dried to yield 1.28 gm (86%) of 1-(4-aminobenzyl)-ethylenediaminetetramethylenephosphonic acid. This compound gave the expected decoupled ^{31}P-NMR spectrum.

This compound may be used as a ligand AP in Formula I where the L is through the NH$_2$ group.

Example N

Preparation of N^4-(p-aminophenyl)-norspermidine

[Bis(cyanoethyl)](p-aminophenyl)amine was prepared as described in US Patent 2,809,985. A 9.0 g (42 mmol) sample of the bisnitrile was dissolved in 90 mL of ethanol and 10 mL of 50% NaOH and treated with 6 g of Raney nickel (w-2 grade) and placed under an atmosphere of hydrogen with shaking. Shaking was continued until th hydrogen uptake

-32-

ceased at which time the catalyst was removed by filtration. The filtrate was reduced in volume under vacuum to a viscous oil, dissolved in 40 mL of 50% NaOH, and extracted with three 75 mL portions of chloroform. The combined chloroform layers were dried over sodium sulfate, decanted and rotoevaporated under vacuum to yield 7.2 gm (77% yield) of the title compound.

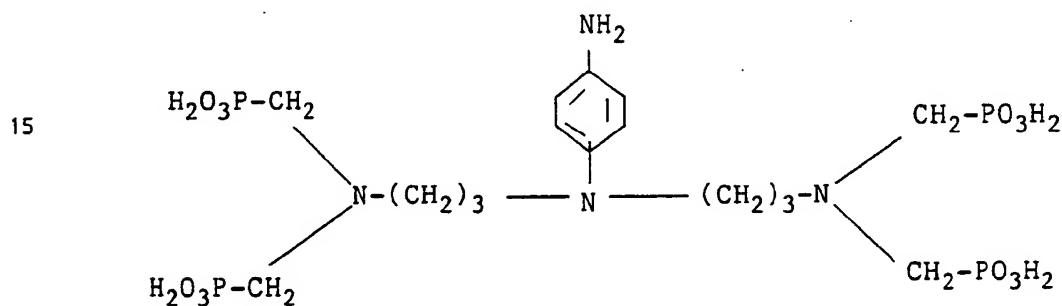5   The product was characterized by proton NMR.

This compound may be used as a ligand AP in Formula I where the L is through the NH$_2$ group.

Example O

Preparation of N''(4-aminophenyl)-dipropylenetriamine-N',N',N''',N'''-

10   tetramethylenephosphonic Acid (APIPMP)

The structure of the compound APIPMP is shown by the formula

15

$$H_2O_3P-CH_2$$
$$\diagdown$$
$$N-(CH_2)_3 \underline{\hspace{1.5cm}} N \underline{\hspace{1.5cm}} (CH_2)_3-N$$
$$\diagup$$
$$H_2O_3P-CH_2$$

with NH_2 substituted phenyl ring connected at N, and $CH_2-PO_3H_2$, $CH_2-PO_3H_2$ on the right nitrogen.

20

N^4-(p-aminophenyl)norspermidine, prepared in Example N (3.65 g, 16.4 µmoles) was added to 25 mL of water and treated with a solution of 6.2 g (75.6 mMoles) phosphorous acid in 13 mL (163 mMoles) of concentrated HCL. The resulting dark blue solution was heated to 100°C and treated dropwise with 5.9 g (73 mMoles) of 37 weight percent formaldehyde in

25   water over a 2.75 hour period. The solution was kept at reflux for an additional 15 hours, then cooled to room temperature and dripped into ethyl alcohol. The resulting brown precipitate was vacuum filtered and dried under vacuum to give 3.84 g (40% yield) of the title compound (APIPMP) as a brown solid. The product thus obtained gave a retention time of 5.50 minutes (minor peak) and 10.50 (major peak) when analyzed on the anion exchange HPLC system
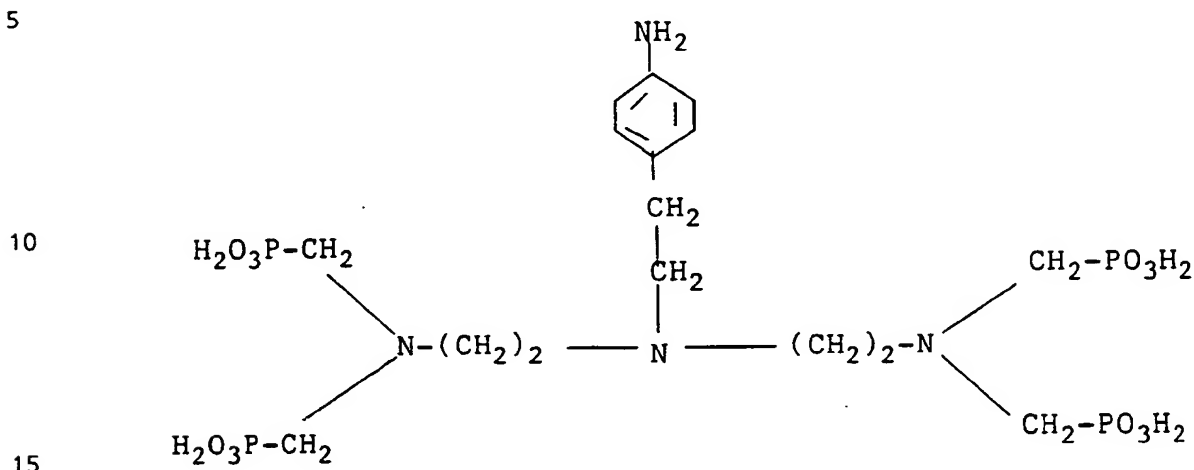
30   described in Example L.

This compound may be used as a ligand AP in Formula I where the L is through the NH$_2$ group.

35

-33-

Example P

Preparation of N''-(4-aminophenethyl)-diethylenetriamine-N',N',N''',N'''-tetra-
methylenephosphonic acid, tetrasodium salt (APDTMP)

The structure of the compound APDTMP is shown by the formula

5

$$
\begin{array}{c}
NH_2 \\
\\
H_2O_3P-CH_2 \quad\quad\quad\quad CH_2 \quad\quad\quad\quad CH_2-PO_3H_2 \\
\\
N-(CH_2)_2 \longrightarrow N \longrightarrow (CH_2)_2-N \\
\\
H_2O_3P-CH_2 \quad\quad\quad\quad\quad\quad\quad\quad CH_2-PO_3H_2
\end{array}
$$

10

15

Diethylenetriamine (20 g, 0.045 mole) was dissolved in 200 mL of toluene and
treated over five minutes with a solution of p-nitrophenethyl bromide (10 g, 0.89 mole) in 150
mL of toluene. After three hours of stirring the supernatent was decanted from the gummy
solid and extracted three times with 100 mL portions of water. The combined water layers

20   were reduced under vacuum to a low volume and back extracted with 100 mL chloroform. The
chloroform was then evaporated under vacuum to yield 9.32 g (83%) of N''-(p-nitrophenethyl)-
diethylenetriamine.

A 5 g sample of this amine was then dissolved in 3N HCl to yield a pH less than 2.
The resulting solution was poured into excess methanol. The resulting precipitate was filtered

25   and dried under vacuum to yield 3 g of the corresponding hydrochloride salt. A 1.3 g (.004
mole) portion of this salt was dissolved in 20 mL of water and treated with phosphorous acid
(1.5 g, 0.018 mole) and concentrated hydrochloric acid (2.0 g; 0.019 moles). The solution was
brought to reflux and 37% formaldehyde (1.5 g, 0.019 mole) was added in a dropwise fashion.
After two additional hours of reflux the solution was cooled to room temperature and

30   evaporated under vacuum to give a viscous oil. This oil was added dropwise to 150 mL of
methanol with vigorous stirring. The resulting white precipitate was filtered and dried under
vacuum to give 1.65 g (66% yield) of N''-(4-nitrophenethyl)diethylenetriamine-N',N',N''',N'''-
tetra-methylenephosphonic acid. In the HPLC method described for Example L this product
exhibited a single peak with retention time of 16.9 minutes.

35   A 700 mg (1.1 mMole) sample of this compound was dissolved in 44 mL (4.4
mMole) of 0.1N NaOH. To this solution was added 100 mg of 10% Pd on carbon suspended in
56 mL water and the whole reaction mixture was placed under hydrogen with vigorous

-34-

shaking. After hydrogen uptake ceased, the catalyst was filtered and the filtrate evaporated
under vacuum to give 0.80 g (100% yield) of N''-(4-aminophenethyl)diethylenetri-amine-
N',N',N''', N'''-tetramethylenephosphonic acid tetrasodium salt (APDTMP) as a hygroscopic
yellow solid. The title compound exhibited a single peak with a retention time of 5.95 minutes
on the HPLC system described for Example L.

This compound may be used as a ligand AP in Formula I where the L is through the
NH_2 group.

Example Q

Preparation of 4-Isothiocyanatophthalic Anhydride

(ACL-3, an acid cleavable linker)
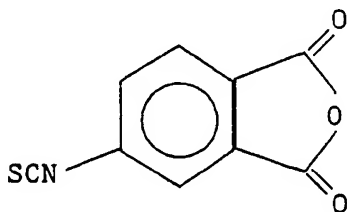
To a slurry of 4-aminophthalic acid (2.7183 grams(g), 15.00 mmol) and anhydrous
potassium carbonate (8.75 g, 63.3 mmol) in 70 mL of tetrahydrofuran (THF) was added
thiophosgene (2.30 mL, 30.18 mmol). The reaction mixture was stirred 10 minutes at ambient
temperature, then heated to reflux for one hour. After cooling to room temperature, the
reaction product solution was filtered through celite and then concentrated to dryness under a
stream of dry nitrogen and under an efficient fume hood to avoid exposure to thiophosgene.
The crude 4-isothiocyanatophthalic acid was thus recovered; 1H NMR (300 MHz, acetone-d6) d
10.22 (br s, 2H), 7.81 (br s, 1H) 7.61 (s,1H), 7.48 (br s, 1H); 13C NMR (75 MHz, acetone-d6)
d 167.8, 167.7, 138.3, 135.8, 134.4, 131.7, 131.7, 128.6, 126.5.

The 4-isothiocyanatophthalic acid thus obtained was immediately heated to
reflux in a mixture of trifluoroacetic anhydride and methylene chloride for 2 hours under a
nitrogen atmosphere. After cooling to room temperature, the reaction mixture was
concentrated under reduced pressure. Recrystallization of the resultant solid from 30 mL of
carbon tetrachloride afforded 4-isothiocyanatophthalic anhydride as brownish-purple crystals
in a yield of 2.3778 g, (77 percent of theoretical). The title compound melted at 106-108°C.; 1H
NMR (300 MHz, acetone-d6) d 8.15 (d, J = 8.1 Hz, 1H), 8.06 (d, J = 1.7 Hz, 1H), 7.97 (dd, J = 8.1, 1.7
Hz, 1H); 13C NMR (75 MHz, acetone-d6) d 163.3, 162.6, 139.8, 134.8, 134.6, 130.6, 128.2, 123.5,
111.6; IR (CHCl₃) 2010 (br), 1840, 1740 cm-1; MS m/e 205, 161, 133 (base), 74.

-35-

FINAL PRODUCTS

Example 1 and Comparative A

Conjugation of PDGF with SCN-BDTMP

A 400 μL portion of 34.7mM SCNBDTMP solution (1.389 x 10^{-5} moles) (prepared in
5   Example G) was added to aliquot 1 from Example E. This steoichometry gives a 794:1 molar
ratio of isothiocyanate group per amino group on the protein (Sample 1).

A 400 μL portion of the 0.3M sodium bicarbonate buffer was added to aliquot 2
from Example E as a control (Comparative A).

Both samples 1 and 2 were mixed well. The samples, still on the membrane, were
10   allowed to set at room temperature for 14.5 hours. The samples were then centrifuged at full
speed to concentrate the samples for one hour. Following centrifugation each sample read
10.0 μCi on the membrane. To each membrane was added 1.0 mL of 0.1M sodium phosphate
buffer. After swirling to mix, the samples were again centrifuged at full speed for 40 min. The
filtrate cups were removed and replaced with new tared filtrate cups. Each sample read 9.9 μCi
15   on the membrane.

To each sample was added 740 μL portion of HSA solution to bring the volume on
the membrane to 800 μL. Each solution was mixed and transferred into new tared filtrate cups.
Each membrane unit was washed with 500 μL of 1% HSA. The Centricon™ units were then
capped, inverted, and centrifuged for about 10 min. at full speed to remove the membrane
20   retentate into the cap. The wash solutions were pipetted into the corresponding filtrate cup.
The Centricon™ caps were also washed with 200 μL portion of HSA solution which was then
transferred to the filtrate cups. These respective final solutions contained about 18.5 μg (1) and
19.0 μg (Comparative A) in 1.42 mL of HSA and 0.15M sodium chloride.

Example 2

25   Conjugation of one SCN-BDTMP per IGF

A 10 μL (3.47 x 10^{-8} mole) portion of 3.47mM SCN-BDTMP solution (prepared in
Example G) was added to Centricon™ no. 1 of Example H (containing approximately 41.2 μg of
IGF). An additional 390 μL of 0.1M sodium bicarbonate buffer at pH = 9.35 was added. The
solution residing on the Centricon™ membrane was then mixed well and allowed to set for 14
30   hours at room temperature. The unit was then reverse spun to remove the membrane's
contents. A 158 μL aliquot of 0.1M sodium bicarbonate was added to bring the final volume to
about 600 μL.

Example 3

Conjugation of two SCN-BDTMP per IGF

35   A 20 μL (6.94 x 10^{-8} mole) portion of 3.47 mM SCN-BDTMP solution (prepared in
Example G) was added to Centricon™ no. 2 of Example H (containing approximately 41.7 μg of
IGF). An additional 390 μL of 0.1M sodium bicarbonate buffer at pH = 9.35 was added. The
solution residing on the Centricon™ membrane was then mixed well and allowed to set for 14

-36-

hours at room temperature. The unit was then reverse spun to remove the membrane's contents. A 86 μL aliquot of 0.1M sodium bicarbonate was added to bring the final volume to about 600 μL.

Example 4

5   Conjugation of four SCN-BDTMP per IGF

A 400 μL (1.39 x 10^{-5} mole) portion of 34.7 mM SCN-BDTMP solution (prepared in Example G) was added to Centricon™ no. 3 of Example H (containing approximately 45.7 μg of IGF). The solution residing on the Centricon™ membrane was then mixed well and allowed to set for 14 hours at room temperature. The unit was then reverse spun to remove the

10   membrane's contents. A 208 μL aliquot of 0.1M sodium bicarbonate was added to bring the final volume to about 600 μL.

Comparative B

Control for Examples 2, 3 and 4

A 400 μL portion of 0.1M sodium bicarbonate buffer at pH = 9.35 was added to

15   Centricon™ no. 4 of Example H (containing approximately 47.7 μg of IGF). An additional 390 μL of 0.1M sodium bicarbonate buffer at pH = 9.35 was added. The solution residing on the Centricon™ membrane was then mixed well and allowed to set for 14 hours at room temperature. The unit was then reverse spun to remove the membrane's contents. A 23 μL aliquot of 0.1M sodium bicarbonate was added to bring the final volume to about 600 μL.

20   Example 5

Conjugation of four SCN-BDTMP per IGF

A 400 μL (1.39 x 10^{-5} mole) portion of 34.7mM SCN-BDTMP solution (prepared in Example G) was added to Centricon™ no. 1 of Example I (containing approximately 130 μg of IGF). The solution residing on the Centricon™ membrane was then mixed well and allowed to

25   set for 13 hours at room temperature. The unit was then centrifuged to concentrate the protein of the solution. The residual on the membrane was then washed with 0.5 mL of 0.1M sodium phosphate buffer (pH = 7.4) by adding buffer and then centrifuging for one hour. The residual on the Centricon™ membrane was then removed by adding 572 μL of a 1 percent HSA solution, swirling and pipetting. The membrane was washed with an additional 0.5 mL of 1

30   percent HSA to completely remove the membrane's contents. The final volume of combined recovered solution was about 1.5 mL containing an estimated 9.5 μg/100 μL of modified IGF in about 1 percent HSA.

Comparative C

Control for Example 5

35   A 400 μL portion of 0.3M sodium bicarbonate buffer at pH = 9.48 was added to Centricon™ no. 2 of Example I (containing approximately 127 μg of IGF). The solution residing on the Centricon™ membrane was then mixed well and allowed to set for 13 hours at room temperature. The unit was then spun to concentrate the protein of the solution. The residual

-37-

on the membrane was then washed with 0.5 mL of 0.1M sodium phosphate buffer (pH = 7.4) by

adding buffer and then spinning on a centrifuge for one hour. The residual on the Centricon™

membrane was then removed by adding 855 μL of a 1% HSA solution, swirrling and pipetting.

The membrane was washed with an additional 0.5 mL of 1% HSA to completely remve the

5   membrane's contents. The final volume of combined recovered solution was about 1.4 mL

containing an estimated 9.5 μg/100 μL in about 1% HSA.

Example 6

IGF modified with BDTMP using ACL-3

            A 175 uL portion of ACL-3 (12.1 mg ACL-3 in 200 uL of trifluoroethanol, prepared

10 in Example Q) was added to a Centricon™ containing approximately 100 μg of IGF in about 500

μL of 0.3M bicarbonate buffer (pH = 9.5). The resulting pH was 8.85. After 15 minutes at room

temperature, the Centricon™ unit was then centrifuged until the volume had dropped to

about 243 μL (243 mg weight) which took about 2 hours. To this concentrated solution was

added 300 μL of ABDTMP solution (7.1 mg, 10.5 μMoles in 300 μL of bicarbonate buffer at

15 pH = 9.5). The resulting pH was 9.27. This solution was allowed to stand at room temperature

for 46 hours. The pH was found to be 9.78 and the volume was about 0.485 μL. This was put on

a centrifuge (Clay-Adams) for 6 hours and the volume was reduced to 117 μL. A 2 mL aliquot of

0.3M sodium bicarbonate buffer was added and the solution again centrifuged. After 10 hours

of centrifuging the volume was about 49 μL. An additional 500 μL of 0.3M sodium bicarbonate

20 was added and the Centricon™ unit centrifuged for 4 hours to give a final volume of 52 μL. A

547 μL portion of 0.1M sodium phosphate buffer (pH = 7.02) was added and mixed well. The

total weight in the Centricon™ unit was 599 mg or about 600 μL in volume. The estimated

concentration in this sample is 100 μg IGF per 600 μL of solution (16.7 μg/100μL).

Comparative D

25 Control for Example 6

            A 175 μL of trifluoroethanol was added to a Centricon™ containing

approximately 100 μg of IGF in about 500 μL of 0.3M bicarbonate buffer (pH = 9.5). The

resulting pH was 9.50. After 15 minutes at room temperature, the Centricon™ unit was then

centrifuged until the volume had dropped to about 230 μL (230 mg weight) which took about

30 2 hours. To this concentrated solution was added 300 μL of 0.3M sodium bicarbonate buffer.

The resulting pH was 9.46. This was allowed to set at room temperature for 46 hours. The pH

was found to be 9.79 and the volume was about 397 μL. This was put on a centrifuge (Clay-

Adams) for 6 hours and the volume was reduced to 27 μL. A 2 mL aliquot of 0.3M sodium

bicarbonate buffer was added and the solution again centrifuged. After 10 hours of

35 centrifuging the volume was about 21 μL. An additional 500 μL of 0.3M sodium bicarbonate

was added and the Centricon™ unit centrifuged for 4 hours to give a final volume of 27 μL. A

573 μL portion of 0.1M sodium phosphate buffer (pH = 7.02) was added and mixed well. The

total weight in the Centricon™ unit was 603 mg or about 600 μL in volume. The estimated IGF concentration in this sample is 100 μg IGF per 600 μL of solution (16.7 μg/100μL).

Example 7

IGF modified with BDTMP using ACL-3

5   A 210 μL aliquot of IGF solution (100 μg of IGF containing tracer amounts of ^{125}I labeled IGF in buffer solution) was put on a membrane of a Centricon™ membrane filtration devices. A 500 μL aliquot of 0.3M sodium bicarbonate was added to both Centricon™ unit and it was then centrifuged to less than 50 mg remaining on the membrane. The contents on the membrane (washed IGF) was taken up in 500 μL more of 0.3M sodium bicarbonate and was

10 treated with the 19.4 mg of ACL-3 dissolved in 300 μL of warm trifluoroethanol. The sample was allowed to stand for 15 minutes then centrifuged for 2 hours. At the end of this time a 300 μL volume of ABHDP (357 μMoles) was added to the Centricon™ unit containing the modified IGF and the solution then allowed to stand for about 16 hours. The pH remained about 10 during this time. The Centricon™ was then centrifuged for about 4 hours to reduce

15 the volume. The membrane retained material (modified IGF) was then diluted with 500 μL of sodium bicarbonate buffer and reconcentrated by centrifuging. The residual membrane retained volume was 221 mg. A 1.279 mL volume of 0.1M sodium phosphate (pH = 7.02) was added to bring the final volume up to 1.5 total. The Centricon™ was then reverse spun to remove the membrane retained material. This material was used in biodistribution studies in

20 rats shown in Example V.

Comparative E

Control for Example 7

          The same procedure was followed as in Example 7 except that the addition of warm trifluoroethanol did not contain the ACL-3 linking compound.

25 Example 8

PDGF modified with BDTMP using ACL-3

          A 100 μL aliquot of PDGF solution was put on the membrane of a Centricon™ membrane filtration device. The PDGF solution contained 10 μCi of ^{125}I labeled PDGF (22 μCi/μg) per 200 μL of 0.1M sodium phosphate, 0.01M sodium citrate, 0.5M NaCl and 1% Bovine

30 Serum Albumin at pH = 4.6. A 500 μL aliquot of 0.3M sodium bicarbonate was added to the Centricon™ unit and it was then centrifuged until 50 mg remained on the membrane. The contents on the membrane (washed PDGF) were taken up in 500 μL more of 0.3M sodium bicarbonate. The membrane retained material was then treated with the 36.7 mg of ACL-3 dissolved in 300 μL of warm trifluoroethanol, allowed to stand for 15 minutes, and then

35 centrifuged for 2 hours. At the end of this time a 300 μL volume of ABHDP (357 μMoles) was added to the Centricon™ unit containing the modified PDGF and the solution then allowed to stand for about 16 hours. The pH remained about 10 during this time. The Centricon™ was then centrifuged for about 4 hours to reduce the volume. The membrane retained material

-39-

(modified PDGF) was then diluted with 500 µL of sodium bicarbonate buffer and reconcentrated by centrifuging. The residual membrane retained volume was 329 mg. A 1.171 mL volume of 0.1M sodium phosphate (pH = 7.02) was added to the membrane of the Centricon™ to bring the final volume up to 1.5 mL total. The Centricon™ was then reverse spun

5 to remove the membrane retained material. This material was used in biodistribution studies in rats as shown in Example VI.

Comparative E

Control for Example 8

The same procedure was followed as in Example 8 except that the addition of

10 warm trifluoroethanol did not contain the ACL-3 linking compound.

BIOLOGICAL EXAMPLES: BIODISTRIBUTION

Example I

^{125}I-PDGF-(SCN-BDTMP)

Spraque-Dawley rats weighing in the 175-230 g weight range were acclimated for

15 5 days prior to injection. The rats were injected via the tail vein with 50 µL of both samples from Example 1 (Samples 1 and Comparative A) (about 200,000 cpm). After 2, 6 and 18 hours, the rats were sacrificed by cervical dislocation, tissues taken, weighed and the amount of radioactivity in each tissue determined by counting for 5 min. in a scintillation counter equipped with a NaI crystal coupled to a multichannel analyzer. The counts in each tissue were

20 compared to the counts in 50 µL standards in order to determine the percentage of injected dose in each tissue. The counts in the tail were subtracted from the standards to give the percent injected dose adjusted for the amount found in the tail. Background counts were obtained and subtracted from the tissue counts. The percent dose in bone was estimated by multiplying the percent dose in the femur by 25. The muscle and blood numbers were

25 obtained assuming that muscle is 43% and blood is 6.5% of the rat body weight. These adjustments of the percent dose for the various body parts are accepted values for the rat model. [W.F. Goeckeler et al., *J of Nucl. Med.* 28(4), 495-504 (1987)]. The results are given in Table I where each data point represents the average of five rats unless noted otherwise.

30

35

Table I
% Injected Dose
Adjusted for Amount of Dose Found in Tail

ORGAN	2 HR.		6 HR.		18 HR.	
	Ex.1*	A●**	Ex.1*	A●	Ex.1**	A●
BONE	22.8	2.8	26.8	2.0	20.9	0.7
LIVER	14.7	12.2	8.1	2.1	2.0	0.5
KIDNEY	16.0	4.3	5.1	1.2	1.6	0.4
SPLEEN	0.7	0.2	0.6	0.1	0.5	0.0
MUSCLE	10.2	5.5	6.6	6.1	8.6	2.3
BLOOD	19.6	2.6	11.7	4.0	4.0	1.0

*average of 3 rats
**average of 4 rats
● is Comparative A

It is readily apparent that conjugation of SCN-BDTMP with PDGF results in targeting of the PDGF to bone. PDGF conjugated to SCN-BDTMP was 8 times more effective in going to bone than native, unmodified PDGF. In addition, the residence time in the bone was increased by about 30 fold.

Example II

^{125}I-IGF-(SCN-BDTMP)

Spraque-Dawley rats weighing in the 175-230 g weight range were acclimated for 5 days prior to injection. The rats were injected via the tail vein with 50 μL of one of the four samples from Examples 2, 3, 4 or Comparative B. After 30 mins. the rats were sacrificed by cervical dislocation, tissues taken, weighed and the amount of radioactivity in each tissue determined by counting for 5 min. in a scintillation counter equipped with a NaI crystal coupled to a multichannel analyzer. The counts in each tissue were compared to the counts in 50 μL standards in order to determine the percentage of injected dose in each tissue. The counts in the tail were subtracted from the standards to give the percent injected dose adjusted for the amount found in the tail. Background counts were obtained and subtracted from the tissue counts. The percent dose in bone was estimated by multiplying the percent dose in the femur by 25. The muscle and blood numbers were obtained assuming that muscle is 43% and blood is 6.5% of the rat body weight. The results are given in Table II where each data point represents the average of five rats.

Table II
% Injected Dose
Adjusted for Amount of Dose Found in Tail

ORGAN	Ex. 2	Ex 3	Ex 4	B*
BONE	9.161	9.688	13.323	6.956
LIVER	5.367	5.318	4.847	3.661
KIDNEY	8.410	11.447	12.756	7.070
SPLEEN	0.380	0.366	0.295	0.350
MUSCLE	19.614	23.440	18.379	26.078
BLOOD	23.909	25.006	19.521	23.169

* Comparative B

The dergee of modification of the IGF with the number of bone seeking groups added via AP of Formula I was based upon stoichiometry of reactants. The results are summarized in Table III where each data point represents the average of five rats.

Table III

Degree of Modification	Example	% Injected Dose	% Increase in Bone uptake
1 group	2	8.5	26
2 groups	3	9.2	36
4 groups	4	11.7	74
none	B*	6.8	0

* Comparative B

Clearly the results demonstrate that targeted delivery of IGF-I to bone can be achieved by the conjugation of SCN-BDTMP to IGF-I. As shown in Table III, the degree of targeting is apparently directly related to the degree of modification.

Example III

^{125}I-IGF-(SCN-BDTMP)

Spraque-Dawley rats weighing in the 175-230 g weight range were acclimated for 5 days prior to injection. The rats were injected via the tail vein with 50 µL of the samples from Examples 5 or C. After 2, 6 and 18 hours the rats were sacrificed by cervical dislocation, tissues taken, weighed and the amount of radioactivity in each tissue determined by counting for 5 min. in a scintillation counter equipped with a NaI crystal coupled to a multichannel analyzer. The counts in each tissue were compared to the counts in 50 µL standards in order to determine the percentage of injected dose in each tissue. The counts in the tail were subtracted from the standards to give the percent injected dose adjusted for the amount found in the tail. Background counts were obtained and subtracted from the tissue counts. The percent dose in

-42-

bone was estimated by multiplying the percent dose in the femur by 25. The muscle and blood
numbers were obtained assuming that muscle is 43% and blood is 6.5% of the rat body weight.
The results are given in Table IV where each data point represents the average of four rats.

Table IV
% Injected Dose
Adjusted for Amount of Dose Found in Tail

ORGAN	2 HR.		6 HR.		18 HR.	
	Ex.5	C*	Ex.5	C*	Ex.5	C*
BONE	16.6	11.1	14.6	9.9	13.4	6.3
LIVER	2.8	5.4	1.4	1.8	0.7	0.9
KIDNEY	4.2	8.4	2.3	2.3	1.1	1
SPLEEN	0.2	0.4	0.2	0.2	0	0.1
MUSCLE	16.9	24.1	11.3	16.5	3.9	3.5
BLOOD	14.3	33.7	7.1	8.7	1.4	1.9

* Comparative C

Example IV

Biological Activity of IGF-I-(SCN-BDTMP)

Various samples from the prior examples were run on an IGF-I mitogenic activity
assay to determine their potency. The protocol is based on a competence-progression model in
which the assay cell line responds to varying levels of IGF-I in a dose dependent manner. Cells
from a mouse fibroblast line are seeded in 96 well assay plates and grown to confluence to
induce quiescence. Prior to addition of samples and standards the cells are preincubated with
PDGF-BB, which enables them to respond to IGF-I when it is added in the presence of EGF.
After an overnight incubation period the cells are exposed to 1.0 μCi of ^{3}H-thymidine which is
incorporated at varying levels depending on the amount of IGF-I added to the assay well.
Following extensive washing, the cells are lysed and the samples are quantitated in a
scintillation counter equipped with a NaI crystal coupled to a multichannel analyzer. The
resulting data are plotted to generate a dose response curve from which unit activity
determinations can be made.

A unit is defined as that concentration of IGF-I which induces 50% of the
maximum cellular response in that assay (ED$_{50}$ value). Maximum response is defined as cellular
response to a 5% FCS standard. Maximum response due to exogenous IGF-I is usually 3-5 times
baseline.

Prior to dilution for the assay, the concentration of IGF-I was determined via an
^{125}I-IGF-I tracer which had been spiked into the stock sample prior to manipulation. Counts
were made on a 25 μL aliquot of the stock sample and 25 μL of the two samples. The ratio of
counts per min (CPM)/μg of IGF-I in the stock sample was used to determine the concentration

-43-

of IGF-I in the two samples for the bioassy. The average of the stock sample aliquots was 5,135 CPM/μg. The values for each sample was determined by CPM per μL and dividing the number by the stock sample aliquot of 5,135 CPM/μg. These measurments and calculations resulted in the following values: Example 5 sample was 107.2 μg/mL; and Example C was 110.8 μg/mL. The

5   results are shown in Table V.

Table V

Example	Sample ED_{50} Avg	Solution Control ED_{50} Avg	% $\frac{\text{Control } ED_{50}}{\text{Sample } ED_{50}}$	% Control
2	3.6	2.2	60	111
3	3.0	2.4	79	146
4	4.8	1.7	35	65
B*	4.3	2.3	54	NA
5	5.6	2.2	39	48
C**	3.8	3.1	82	NA

* Comparative B;
** Comparative C

Example V

^{125}I-IGF-ACL-3-BDTMP)

20   Doses (150 μL) of the material from Example 7 and Comparative Example E were drawn up into 500 μL syringes equipped with 28 gauge needles and injected into the tail veins of 5 rats. After 6 hours the rats were sacrificed and dissected to obtain the organ distribution of the radiolabelled material. Because of the low amount of radioactivity involved, the samples were counted for 10 minutes. The final number of counts (less background) found in the

25   femurs of rats injected with the modified IGF material of Example 7 were 3.8 times the number of counts (less background) found in the femurs of rats injected with the control material of Comparative E as seen in the following Table VI.

30

35

-44-

Table VI

Rat	Example 7	Comparative E
1	673	376
2	1714	383
3	1250	281
4	1409	264
5	1408	375
Average	1291	336
Ratio	3.8	1

Example VI

^{125}I-PDGF-ACL-3-BDTMP)

Doses (100uL) of this material were drawn up into 500 uL syringes equipped with 28 gauge needles and injected into the tail veins of 5 rats for each of Centricon 1 (Experimental) and Centricon 2 (Control). After 6 hours the rats were Aced and dissected to obtain the organ distribution of the radiolabelled material. Because of the low amount of radioactivity involved, the samples were counted for 10 minutes. The final number of counts (less background) found in the femurs of rats injected with Centricon 1 (experimental) material were 3.8 times the number of counts (less background) found in the femurs of rats injected with Centricon 2 (control) material as seen in the following Table VII.

Table VII

Rat	Example 8	Comparative F
1	1535	683
2	750	807
3	1708	859
4	1559	525
5	1543	639
Average	1419	703
Ratio	2	1

The data thus demonstrates that modification of GF's using techniques described in the present invention results in targeted delivery of biologically active GF's to bone.

Although the invention has been described with reference to its preferred embodiments, those of ordinary skill in the art may, upon reading and understanding this disclosure, appreciate changes and modifications which may be made which do not depart from the scope and spirit of the invention as described above or claimed hereafter.

-45-

WHAT IS CLAIMED IS:

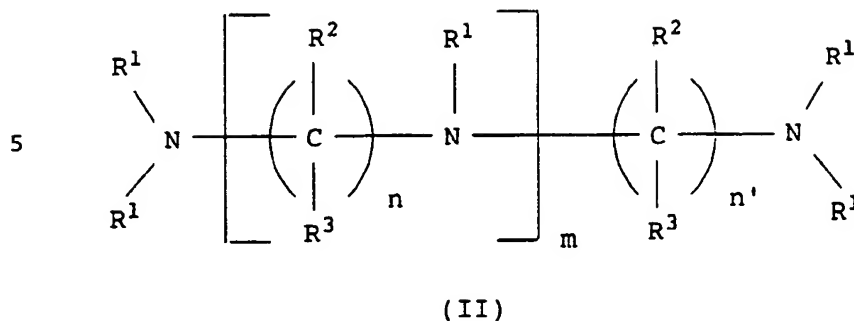1.   A compound of the formula

$$GF-[(CL)_z-L-AP]_q \quad (I)$$
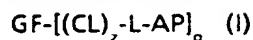
wherein:

5   GF is a tissue growth promoting factor or combinations thereof;

CL is an acid cleavable linker which is covalently bonded to GF;

z is 0, 1 or 2;

q is from 1 to the sum of the amino groups present on the native GF;

L is a linking moiety; and

10   AP is a polyaminomethylenephosphonic acid ligand.

2.   The compound of Claim 1 wherein GF is platelet-derived growth factors, insulin-like growth factors, fibroblast growth factors, epidermal growth factors, transforming growth factors, nerve growth factors or cartilage/bone inductive factors or combinations thereof.

15   3.   The compound of Claim 2 wherein GF is platelet-derived growth factors, insulin-like growth factors, fibroblast growth factors, transforming growth factors, or cartilage/bone inductive factors or combinations thereof.

4.   The compound of Claim 2 wherein GF is the combination of either platelet-derived growth factor and insulin-like growth factor-I or platelet-derived growth factor and
20   insulin-like growth factor-II.

5.   The compound of Claim 1 wherein AP is a ligand that is a straight or branched-chain moiety, cyclic moiety, polymer, or aryl moiety, which ligand contains at least two nitrogen atoms.

6.   The compound of Claim 5 wherein the polymer is a dense star polymer.

25   7.   The compound of Claim 6 wherein the dense star polymer is a dendrimer or dendron.

8.   The compound of Claim 5 wherein the ligand contains three or more nitrogen atoms.

9.   The compound of Claim 5 wherein AP is of the formula

30

35

-46-

$$R^1 \begin{matrix} \\ N \\ \\ \end{matrix}_{R^1}^{R^1} - \left[\left(\underset{R^3}{\overset{R^2}{\underset{|}{\overset{|}{C}}}} \right)_n - \underset{R^1}{\overset{}{N}} - \right]_m \left(\underset{R^3}{\overset{R^2}{\underset{|}{\overset{|}{C}}}} \right)_{n'} - \underset{}{N} \begin{matrix} R^1 \\ \\ R^1 \end{matrix}$$

(II)

wherein:

each R^1 independently is hydrogen, C_1-C_4 alkyl, phenyl, hydroxy C_1-C_4 alkyl, -CH_2COOH, -$CH_2PO_3H_2$ or an L moiety;

with the proviso that only one of R^1 may be an L moiety and one L moiety must be present and with the proviso that at least one-half of the total R^1's are -$CH_2PO_3H_2$;

each R^2 and R^3 independently is hydrogen or C_1-C_4 alkyl or L moiety;

with the proviso that only one L moiety is present in Formula II;
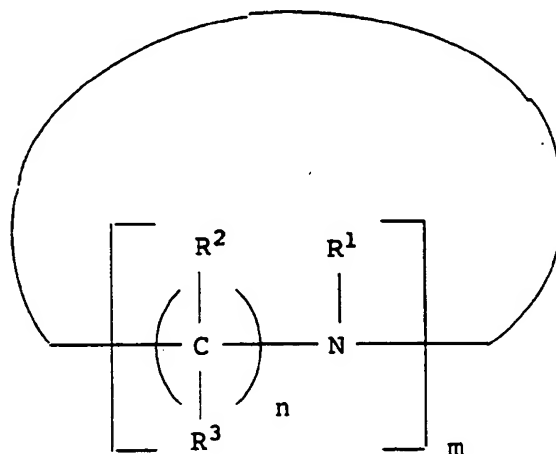
n is 2, 3 or 4;

n' is 2, 3 or 4; and

m is 0 to 10.

10.   The compound of Claim 5 wherein AP is of the formula

$$\left[\left(\underset{R^3}{\overset{R^2}{\underset{|}{\overset{|}{C}}}} \right)_n - \underset{R^1}{\overset{}{N}} \right]_m$$

(III)

-47-

wherein: R^1, R^2, R^3, n and m are defined as in Claim 9.

    11.   The compound of Claim 5 wherein AP is a straight or branched-chain
moiety.

    12.   The compound of Claim 11 wherein the straight or branched-chain moiety
is

    (N-propylcarboxyl)ethylenediamine-N,N',N'-trimethylenephosphonic acid;

    [N-(4-aminophenyl)ethyl]ethylenediamine-N,N',N'-trimethylenephosphonic acid;

    1-(carboxyl)ethylenediamine-N,N,N',N'-tetramethylenephosphonic acid;

    [1-(4-aminobenzyl)]ethylenediamine-N,N,N',N'-tetramethylenephosphonic acid;

    N-(4-aminophenyl)-N,N-bis-[propyl(iminodimethylenephosphonic acid)];

    N-[(4-aminophenyl)ethyl]-N,N-bis-[ethyl(iminodimethylenephosphonic acid)]; or

    N-[1-(4-aminobenzyl)-N,N'-ethylenediamine-N',N''-ethylenediamine-N,N,N',N''-
pentamethylenephosphonic acid.

    13.   The compound of Claim 5 wherein AP is a cyclic moiety.

    14..   The compound of Claim 13 wherein the cyclic moiety is

    1,4,7,10-tetraazacyclododecane;

    1,5,8,12-tetraazacyclotetradecane;

    2-[(4-aminobenzyl)-1,4,7,10-tetraazacyclododecane]-1,4,7,10-tetramethylene-
phosphonic acid;

    1-[(α-carboxyl)-4-amino-2-methoxybenzyl]-1,4,7,10-tetraazacyclododecane-
4,7,10-trimethylenephosphonic acid; or

    1-[(α-phosphonyl)(4-aminophenyl)ethyl]-1,4,7,10-tetraazacyclododecane-4,7,10-
trimethylenephosphonic acid.

    15.   The compound of Claim 5 wherein AP is a aryl moiety.

    16.   The compound of Claim 15 wherein the aryl moiety is an aromatic ring
system having a total number of atoms in the backbone of the aryl ring is from 3 to 30.

    17.   The compound of Claim 16 wherein the aromatic ring system has one or
more additional cyclic or aromatic rings present, or is substituted by straight or branched-chain
moieties.

    18.   The compound of Claim 16 wherein the aromatic ring system is

    3,6,9,15-tetraazabicyclo[9.3.1]pentadeca-1(15),11,13-triene-3,6,9-trimethylene-
phosphonic acid;

    6-(α-carboxyl-4-aminobenzyl)-3,6,9,15-tetraazabicyclo[9.3.1]pentadeca-
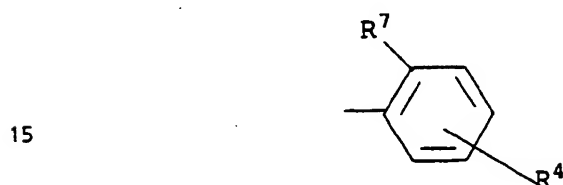1(15),11,13-triene-3,9-dimethylenephosphonic acid;

    13-(4-aminobenzyl)-3,6,9,15-tetraazabicyclo[9.3.1]pentadeca-1(15),11,13-triene-
3,6,9-trimethylenephosphonic acid; or

    6-[(α-phosphonyl-4-aminophenyl)ethyl]-3,6,9,15-tetraazabicyclo[9.3.1]pentadeca-
1(15),11,13-triene-3,9-dimethylenephosphonic acid.

19.   The compound of Claim 1 wherein L is of the formula
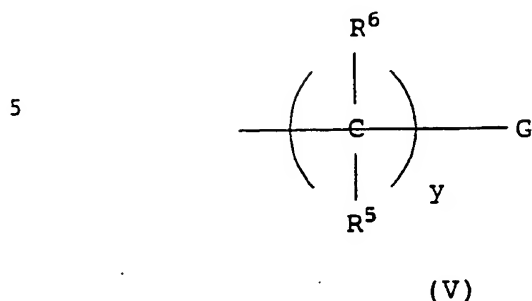
$$R^6$$
$$-\left(\underset{\underset{R^5}{|}}{\overset{\overset{|}{C}}{}}\right)_y - G$$

(V)

wherein: G is hydrogen, NH_2 or

$$R^7$$

$$R^4$$

R^4 is an electrophilic group capable of being attached to protein;

        R^5 and R^6 are independently hydrogen or -COOH;

with the proviso that when G is hydrogen, then one of R^5 or R^6 is COOH;

        R^7 is hydrogen, hydroxy or C_1-C_4 alkoxy; and

        y is 0, 1, 2, 3 or 4;

with the proviso that when y is 1, 2, 3 or 4, then only one of R^5 or R^6 may be COOH.

      20.   The compound of Claim 1 wherein the L moiety is covalently bonded to the GF.

      21.   The compound of Claim 1 wherein z is 1 or 2 and CL is a thiourea, thioether, peptide, ester, disulfide, amide, diester, thioether, hydrocarbon, acetal glycoside or 4-isothiocyanatophthalic anhydride.

      22.   The compound of Claim 1 wherein the L-AP moiety is 1-(α-carboxyl-2-methoxy-5-aminobenzyl)-1,4,7,10-tetraazacyclododecane-4,7,10-trimethylenephosphonic acid.

      23.   The compound of Claim 1 wherein the L-AP moiety is 1-(4-isothiocyanatobenzyl)diethylenetriaminepentamethylenephosphonic acid.

      24.   The compound of Claim 23 wherein the GF is platelet-derived growth factors, insulin-like growth factors, fibroblast growth factors, transforming growth factors, or cartilage/bone inductive factors or combinations thereof.

      25.   The compound of Claim 23 wherein the GF is platelet-derived growth factors or insulin-like growth factors or combinations thereof.

26. A pharmaceutical formulation which comprises a compound of the formula

$$GF\text{-}[(CL)_z\text{-}L\text{-}AP]_q \quad (I)$$
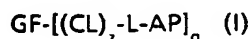
wherein:

GF, CL, L, AP, z and q are as defined in Claim 1; and which is in solid or liquid form; and

5   a pharmaceutically-acceptable carrier or adjuvant.

27. The formulation of Claim 26 which is supplied as a two or more substance in kit form.

28. The formulation of Claim 26 which is supplied as single substance.

29. A method of regenerating bone of a mammal by administering to the

10   mammal in need of such treatment, either by direct application to the area of injured or depleted bone or by indirect application, an effective amount of a composition that includes as the active ingredient a compound of the formula

$$GF\text{-}[(CL)_z\text{-}L\text{-}AP]_q \quad (I)$$

wherein:

15   GF, CL, L, AP, z and q are as defined in Claim 1.

30. The method of Claim 29 wherein the effective amount is from about 0.005 to 50 mg/kg of body weight of the mammal.

31. The method of Claim 29 wherein more than one compound of Formula I is administered.

20   32. The method of Claim 29 wherein other active compounds are also administered.

33. The method of Claim 29 wherein further bone loss is prevented or lessened.

25

30

35

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(5)   :A61K 37/02
US CL   :514/12

According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/12, 514/7, 514/2, 530/399, 530/324

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JP, A, 2-268190 (Fujisaki et al.) 01 November 1990, see entire document.	1-33
A	US, A, 5,011,913 (Benedict et al.) 30 April 1991, see entire document	1-33
A,P	US, A, 5,208,219 (Ogawa et al.) 04 May 1993, entire document, especially col 6, lines 39+.	1-33
A,P	EP, A, 0,512,844 (Bentz et al.) 11 November 1992, see entire document.	1-33

☐ Further documents are listed in the continuation of Box C.   ☐   See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
01 SEPTEMBER 1993	08 SEP 1993

Name and mailing address f the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer DAVID LUKTON
Facsimile N . NOT APPLICABLE	Telephone N . (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

DIALOG
(phosphonate or aminophosphonic or polyamin methylenephosphonic or ph sph nic)
(growth factor or peptide or polypeptide)
(bone or osteoporosis)
(affinity or binding or coordinate or localize or seeking)